UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS
DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

FABRÍCIO EULÁLIO LEITE CARVALHO

CHLOROPLAST REDOX METABOLISM AND NPQ REGULATION IN ARABIDOPSIS AND RICE EXPOSED TO EXCESSIVE LIGHT – NEW INSIGHTS INTO PSII PHOTOPROTECTION MECHANISMS

FORTALEZA
2016

FABRÍCIO EULÁLIO LEITE CARVALHO

CHLOROPLAST REDOX METABOLISM AND NPQ REGULATION IN ARABIDOPSIS AND RICE EXPOSED TO EXCESSIVE LIGHT – NEW INSIGHTS INTO PSII PHOTOPROTECTION MECHANISMS

Tese apresentada ao programa de pós-graduação em Bioquímica da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Doutor em Bioquímica. Área de concentração: Bioquímica vegetal.

Orientador: Prof. Dr. Joaquim Albenísio Gomes da Silveira (UFC-BRA)
Co-orientador: Prof. Dr. Alexander V. Ruban (QMUL-UK)

FORTALEZA
2016

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca Universitária
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

C323c Carvalho, Fabrício Eulálio Leite.
Chloroplast redox metabolism and npq regulation in arabidopsis and rice exposed to excessive light : new insights into psii photoprotection mechanisms / Fabrício Eulálio Leite Carvalho. – 2016.
171 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica , Fortaleza, 2016.
Orientação: Prof. Dr. Joaquim Albenísio Gomes da Silveira.
Coorientação: Prof. Dr. Alexander V. Ruban.

1. Alta luz. 2. APX tilacoidal. 3. H202. 4. Oryza sativa. 5. Quenching não-fotoquímico. I. Título.
CDD 572

FABRÍCIO EULÁLIO LEITE CARVALHO

CHLOROPLAST REDOX METABOLISM AND NPQ REGULATION IN ARABIDOPSIS AND RICE EXPOSED TO EXCESSIVE LIGHT – NEW INSIGHTS INTO PSII PHOTOPROTECTION MECHANISMS

Tese apresentada ao programa de pós-graduação em Bioquímica da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Doutor em Bioquímica. Área de concentração: Bioquímica vegetal.

Aprovada em 07 / 07 / 2016

BANCA EXAMINADORA

Prof. Dr. Joaquim Albenísio Gomes da Silveira (Orientador)
Universidade Federal do Ceará (UFC)

Prof. Dr. Claudivan Feitosa de Lacerda
Universidade Federal do Ceará (UFC)

Prof. Dr. Evandro Nascimento da Silva
Universidade Estadual do Ceará (UECE)

Prof. Dr. Danilo de Menezes Daloso
Universidade Federal do Ceará (UFC)

Prof. Dr. Milton Costa Lima Neto
Universidade Estadual Paulista (UNESP)

Agradecimentos (Acknowledgements)

Ao Prof. Dr. Joaquim Albenísio Gomes da Silveira, por orientar-me durante toda minha jornada científica, desde a graduação até a conquista do doutorado. Por tantos sábios conselhos e por todas as grandes oportunidades que sempre me concedeu. Obrigado.

To Prof. Dr. Alexander V. Ruban for promptly receive me in your excellent group, thank you for open a new world of science to me. The way I see the world will never be the same. Thank you.

Ao Prof. Dra. Marcia Margis-Pinheiro, por receber-me em Porto Alegre diversas vezes e tantos experimentos envolvendo as plantas transformadas de arroz. Por sempre manter uma porta aberta para mim e por tantas valiosas oportunidades. Obrigado.

A todos os membros convidados da banca avaliadora, por sua colaboração e disponibilidade.

À minha amiga Ana Karla M. Lobo por tantas vezes me ajudar com os experimentos e não me deixar esquecer os prazos e as provas. Obrigado.

Ao meu amigo Prof. Dr. Marcio O. Martins, por sempre me ajudar em todos os experimentos, correções e todo o trabalho de bancada. Por suas valiosas dicas com IRGA. Obrigado.

Ao meu amigo Prof. Dr. Milton C. Lima Neto, por sempre me ajudar a planejar os experimentos e por todo seu apoio na bancada. Obrigado por seus ensinamentos de fluorimetria.

To my friend Dr. Erica Belgio, for help so much in London, for discuss my data and teach me so many times. Thank you.

To my friend Max Ware, for promptly help in QMUL arriving, for help in the experiments discussion and manuscript writing. Cheers mate!

To my friend Dr. Vasco Giovagnetti for help me in the lab work and keep so good working environment. Grazie amico!

To dear Dr. Petra Ungerer, for help me so many times in lab work and being always so kind. Thank you.

A todos os meus companheiros de LABPLANT, especialmente João Víctor, Lara, Juliana e Adilton, sempre acessíveis e que tanto colaboraram por toda a jornada. Obrigado amigos.

To all my friends in QMUL, especially Emma, Sunny, Jun, Amy, Yonglan, Ruth and Chris, for always make me fell in home. Thank you.

A todos os colegas estudantes e pós-docs da UFRGS, os quais sempre colaboraram com as pesquisas desenvolvidas durante toda minha formação acadêmica.

Ao Prof. Dr. Sergio Ferreira-Silva por ter me ajudado nos primeiros passos do laboratório e durante toda a jornada. Muito obrigado.

Ao Prof. Dr. André L. Silva por sempre manter uma porta aberta e tantas vezes colaborar com meu trabalho. Obrigado por tantas oportunidades.

À Prof. Dra. Aurenívia Bonifácio por toda colaboração e auxílio, especialmente durante os meus primeiros anos de iniciação científica.

A todos os professores do DBBM que colaboraram muito para minha formação acadêmica especialmente ao Prof. Dr. José Tadeu Abreu de Oliveira e Prof. Dr. Francisco de Assis Paiva Campos. Muito obrigado.

A todos os colegas do DBBM que sempre me ajudaram nos momentos de necessidade. Obrigado.

A todo o pessoal da limpeza e administração que sempre fazem o melhor de seus esforços para que possamos trabalhar em um ambiente agradável e funcional.

À minha querida noiva e futura esposa Luana por sempre ser tão paciente e compreensiva com meus esforços em prol desse objetivo que foi o título de doutor. Obrigado por sempre me apoiar e confortar-me nas horas em que tudo parecia impossível e desesperador. Obrigado por tanta paciência e serenidade mesmo com os longos meses de distância em outro país. Divido essa conquista com você, amor da minha vida.

Aos meus amados pais Aloizio e Betânia que sempre me apoiaram por toda vida. Foi deles que recebi as primeiras lições do mundo e as primeiras palavras de conforto. Graças a vocês pude perceber ainda muito jovem o quanto o mundo era grandioso e como era preciso aproveitar ao máximo o que ele tinha a oferecer. Testemunhei muitas horas de dificuldades e sacrifícios, mas, acima de tudo, serenidade e amor para sempre me oferecer o melhor que podiam. Vocês me deram a vida e o mundo! Muito obrigado.

À Deus e Nossa Senhora de Fátima, que sempre iluminaram meu caminho e me trouxeram saúde e coragem para trabalhar.

"Não importa a altura da montanha ela nunca será grande o suficiente para bloquear o sol."

Provérbio chinês

RESUMO

Plantas são organismos sésseis e por sua natureza desenvolveram diversos mecanismos ligados a maximização da utilização dos recursos primordiais disponíveis para sua sobrevivência nas mais diversas condições ambientais. Em condições ambientais ótimas de crescimento, a intensidade de energia absorvida a partir da luz incidente equivale a demanda energética exercida pela atividade metabólica circunstancial da planta. Entretanto, na quase totalidade dos casos, tais condições não existem na natureza. O excesso de luz é caracterizado por uma condição ambiental na qual o organismo vegetal é submetido a uma quantidade de energia luminosa mais alta do que a capacidade de utilização nas reações bioquímicas que compõem o metabolismo celular necessário para o crescimento normal do indivíduo. Sendo assim, o excesso de luz pode ser consequência de altas intensidades de luz incidente ou devido a restrições nas reações de consumo de poder redutor, especialmente assimilação de CO_2. Portanto, plantas submetidas a estresse salino por exemplo, o qual está muitas vezes associado a uma restrição de natureza estomática, podem apresentar estresse de excesso de luz, mesmo sob intensidades moderadas de luz incidente. A energia excedente pode aumentar a probabilidade da ocorrência de reações colaterais tais como a formação das espécies reativas de oxigênio (EROS). Essas moléculas de alta reatividade podem gerar diversos efeitos negativos para o funcionamento celular, incluindo desnaturação de proteínas, peroxidação lipídica de membranas e fotoinibição das atividades do fotossistemas II (PSII). A ocorrência de fotoinibição é extremamente negativa para o crescimento e desenvolvimento vegetal, afetando a produção de alimentos global. Entretanto, pressões evolutivas causadas pela seleção natural conduziram ao aparecimento de diversos mecanismos de proteção fotooxidativa. Entre as respostas de fotoproteção acionadas em vegetais, duas merecem especial destaque: a dissipação de energia na forma de calor (componente qE do *quenching* não-fotoquímico - NPQ) e a modulação da expressão de genes ligados a rotas de síntese de antioxidantes enzimáticos e não enzimáticos. Em uma perspectiva sistêmica, entretanto, nenhuma dessas respostas ocorre de maneira isolada em uma célula vegetal. Contrariamente, esses mecanismos interagem de maneira contínua e interdependente, partilhando substratos e rotas de sinalização, e, deste modo, conduzindo à aclimatação da planta às condições de excesso de luz.

O presente estudo de doutorado objetivou primariamente aprofundar os conhecimentos existentes acerca dos papéis do NPQ na fotoproteção de plantas. Com esse intuito, plantas modelo da espécie *Arabidopsis thaliana* L. foram utilizadas para entender as dinâmicas fotossintéticas em resposta ao excesso de luz. Uma metodologia pioneira para a detecção de fotoinibição, baseada em técnicas de fluorimetria da clorofila *a* relacionadas à quantificação do *quenching* fotoquímico medido no escuro, subsequente a um período de iluminação (qPd) foi utilizada. Essa metodologia permite a quantificação dos primeiros indícios de fechamento permanente dos centros de reação do PSII (RCII) e possibilita a quantificação da fração de NPQ que de fato contribui para a fotoproteção (pNPQ). Plantas de Arabidopsis de diferentes idades foram utilizadas, objetivando-se a aplicação da metodologia em um problema fisiológico relevante. Os resultados obtidos nesse estudo são apresentados no capítulo 2 do pressente trabalho e evidenciam que: a) plantas de Arabidopsis apresentam maior susceptibilidade a fotoinibição nas fases juvenil e senescente da ontogênese, b) a fotoproteção foi positivamente associada com a maior eficiência de formação do pNPQ e conteúdo total de clorofilas; c) a maior susceptibilidade à fotoinibição coincidiu com o acúmulo de EROS nas fases juvenil e

senescente e d) o uso da técnica baseada no qPd foi bem sucedido, evidenciando o potencial para futuras aplicações em outros contextos eco-fisiológicos.

Posteriormente, buscou-se um maior entendimento acerca dos mecanismos inerentes ao parâmetro pNPQ. Utilizou-se uma abordagem simplificada consistindo em apenas três fases ontogenéticas de plantas de Arabidopsis no intuito de investigar os processos de conhecido envolvimento com a formação do qE, ∆pH, relação violoxantina/zeaxantina e quantidade da proteína PsbS. Esses dados estão relacionados no capítulo 3 do presente trabalho e evidenciam que: a) plantas senescentes e juvenis apresentam menor quantidade de PsbS e formação de ∆pH respectivamente em comparação a plantas maduras, b) as plantas maduras apresentam maior quantidade de pigmentos xantofilas que, em combinação com maiores níveis de clorofila reportados anteriormente, podem explicar uma maior eficiência e potencial na formação de qE, o qual estaria intimamente ligado ao pNPQ.

Finalmente, o presente trabalho de doutorado buscou a aplicação dos conhecimentos gerados acerca das novas técnicas de detecção da fotoinibição e pNPQ em uma planta modelo de interesse agronômico, arroz (*Oryza sativa*). Objetivando-se investigar a relação das EROS com os processos de fotoproteção associados ao pNPQ, plantas de arroz silenciadas por meio de RNAi para a expressão das APX de tilacóides (APX8) foram empregadas. Os resultados obtidos nesses estudos se encontram no capítulo 4 da presente tese e evidenciam que: a) em luz moderada, plantas silenciadas (*apx8*) apresentam maior susceptibilidade a fotoinibição associada a menor acúmulo de biomassa e menor produtividade, b) essas características das plantas *apx8* provavelmente estiveram ligadas a menor eficiência de formação de pNPQ e qE, c) as diferenças relacionadas ao pNPQ provavelmente estiveram associadas com menor conteúdo de clorofilas e maior acúmulo de EROS nas plantas *apx8*, evidenciando novamente a importância desses fatores com a capacidade de fotoproteção em plantas e d) durante a aclimatação ao excesso de luz, a ausência da APX8 é compensada por outras peroxidases, levando a redução nos níveis de EROS e provavelmente recuperação dos níveis de clorofila, que levam a uma recuperação do fenótipo evidenciado em luz moderada.

Conclui-se com a presente tese que o uso das metodologias relacionadas a determinação do qPd para estudos de integridade dos RCII foi eficaz para duas espécies distintas, *Arabidopsis thaliana* e *Oryza sativa*. Adicionalmente, os resultados indicam que a eficiência de formação do pNPQ parece ser um fator determinante para a capacidade de fotoproteção, que por sua vez é dependente do conteúdo de pigmentos fotossintéticos, especialmente clorofila e carotenoides, assim como eficiência do metabolismo antioxidativo. Essas relações são primeiramente descritas no presente trabalho de doutorado e apresentam potencial de aplicação futura em estudos envolvendo obtenção de plantas mais tolerantes ao excesso de luz. Tais condições de excesso de luz são recorrentes em diversas regiões do mundo, mas especialmente importantes em regiões do semiárido do nordeste do Brasil, onde se encontram em combinação com outros fatores de estresse abiótico, tais como seca e alta salinidade do solo. Espera-se com essas descobertas, contribuir para o conhecimento científico da área de bioquímica vegetal e, posteriormente, possibilitar sua aplicação em âmbito do desenvolvimento agrícola regional.

Palavras-chave: Alta luz, APX tilacoidal, H_2O_2, *Oryza sativa*, quenching não-fotoquímico.

Abstract

Plants are sessile organisms, which evolved from aquatic environment to land. This crucial change in habitat opened a new word of resources, but also generated numerous adaptive challenges. Among these defies, sustain photosynthesis under an excessive light environment consist in one of the most important adverse factors to be overcame by plants. Excessive light is defined as the light absorbed by plants, which exceeds their photosynthetic capacity. Under such conditions, the over-accumulation of oxygen reactive species (ROS) could lead to impairment on photosystem two reaction centers (RCII), generating photoinhibition and affecting negatively crop productivity. In this sense, the development of photoprotective mechanisms was essential for plants surviving. Among such mechanisms, non-photochemical quenching (NPQ) and antioxidative metabolism have been intensively studied. However, the real importance of such photoprotective mechanisms and the possible integrative processes which connect NPQ and redox metabolism still not totally understood. The present study addressed to comprehend the integrative mechanisms underlying NPQ and antioxidative processes in two different plants species: one classic plant model (*Arabidopsis thaliana*) and one important crop model (*Oryza sativa*). In order to reach such objectives, first was employed new fluorimetric-based methodologies related to photoinhibition quantification, involving photochemical quenching measured in the dark subsequently illumination (qPd) and the fraction of NPQ formed which was related to photoprotection (pNPQ). The use of this methodology was well succeed with *A.Thaliana* plants under an important physiological problem: light tolerance during plant ontogenesis. The importance of ROS accumulation during less tolerant developmental phases (juvenile and senescent) and chlorophyll concentrations were discussed. Subsequently, the mechanisms underlying pNPQ formation were investigated in the same model, which revealed the importance of combined adjustments in PsbS protein expression, delta pH formation and zeaxanthin/violaxanthin pigment contents for phototolerance during *A. thaliana* ontogenesis. The importance of total xanthophyll pool in pNPQ generation was evaluated. Finally, the current study attempted to employ the methodology involving qPd in a crop model (rice) knockdown for thylakoidal ascorbate peroxidase isoform (APX8 proteins; *apx8* = transformed plants). The data revealed new importance for APX8, which were more sensitive to light, specially under moderate growth light (400 μmol m^{-2} s^{-1}) compared with non-transformed (NT). Transformed *apx8* plants accumulated less chlorophyll, over-accumulated H_2O_2 and presented impairment on thermal component of NPQ (qE) formation. Under high light intensities growth conditions (4 days), *apx8* plants activated compensatory mechanisms which were probably effective as photoprotective mechanism.

Key-words: High light, thylakoidal APX, H_2O_2, *Oryza sativa*, non-photochemical quenching.

FIGURES AND TABLES LIST

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

ABBREVIATION LIST

2-DE: two dimensional electrophoresis
apx8: tAPX silenced rice plants
ASC: ascorbate
CAT: catalase
chl: chlorophyll
Ci: internal CO_2 concentration
D1: PsbA encoded gene product
DHA: dehydroascorbate
E: foliar transpiration
ETR: taxa de transporte de elétrons do PSII
FPLC: fast protein liquid chromatography
Fv/Fm: maximum potential PSII quantum efficiency
GO: glycolate oxidase
gs: stomatal conductance
GSH: reduced glutathione
GSSG: oxidized glutathione
H_2O_2: hydrogen peroxide
HPLC: high pressure liquid chromatography
lhcb: major antennas b proteins genes
LHII: PSII light harvesting complex
MDHA: monodehydroascorbate
NPQ: non-photochemical quenching
NT: non transformed plants
OEC: oxygen evolution complex
PETC: photosynthetic electron transport
pNPQ: protective NPQ
PQ: plastoquinone
PQH_2: double reduced plastoquinone
PRX: peroxiredoxins
PsbS: PSII subunit S protein
PSI: photosystem I
PSII: photosystem II
Q_A: PSII strongly bound quinone
Q_B: PSII weakly bound quinone
qP: photochemical quenching (Puddle model)
qPd: photochemical quenching measured in dark after illumination period
RCII: PSII reaction center
RNAi: interference RNA
ROS: reactive oxygen species
Rubisco: Ribulose 1,5 bisphosphate carboxylase/oxygenase
sAPX: stromal ascorbate peroxidase
SOD: superoxide dismutase
tAPX: thylakoidal ascorbate peroxidase
VDE: violaxanthin de-epoxidase
Viol: violaxanthin
Zea: zeaxanthin
$\Delta F/Fm'$: actual PSII quantum efficiency (ΦPSII)
ΔpH: lumen-stromal pH gradient

SUMARY

OBJECTIVES

Plants exposed to excessive light needed to develop several mechanisms of photoprotection as a response of evolution pressures. Therefore, in order to reach new insights into PSII photoprotection mechanisms, the current thesis had the following objectives:

1. Perform a deep and integrative review in order to understand the current state-of-the-art in terms of photoprotection of PSII reaction centers;
2. Employ new methodologies related to PSII photoinhibition monitoring and NPQ study and, subsequently, associate these results with oxidative stress;
3. Compare the results obtained with the use of these new methodologies with classical methodologies regarding qE-related processes.
4. Employ the new knowledge obtained for tracking the PSII photoinhibition to better understanding the importance of thylakoidal ascorbate peroxidases in rice crop models.

CHAPTER I

Review

How plants support photosynthesis under excess light? An integrated view of photoprotective mechanisms.

Fabricio E. L. Carvalho

Department of Biochemistry and Molecular Biology, Federal University of Ceará, Fortaleza/CE – Brazil, Zip Code 60451-970.

1. Introduction

Plants are sessile organisms, which evolved from aquatic environment to land at least 475 million years ago (Wellman et al., 2003). This crucial change in habitat opened to plants a new word of resources, but also generated numerous adaptive challenges (Goss and Lepetit, 2014). Among these defies, sustain photosynthesis under an excessive light environment consist in one of the most important adverse factors to be overcame by plants (Ruban, 2015). Indeed, plants are exposed to a wide variation in light intensity incidence, which are main provoked by occasional shadowing (cloud movements, canopy competition, etc.) and seasonal variations (Raven, 1984). Nevertheless, for several plants species excessive light is a very usual condition.

Excessive light is defined as the light absorbed by plants, which exceeds their photosynthetic capacity (Li et al., 2009a). This concept is very distinct from high light, which in turns, is conditional, relative and restricted to a comparison between different light regimes, and, therefore, is not suitable to define a stressful condition. Indeed, excessive light can occur even under relatively low values of photosynthetic photon flux density (PPFD), according to studied species and circumstantial metabolic demand (Silveira and Carvalho, 2016). Excessive light conditions could generate high levels of reduced power in thylakoid membranes, which can lead to photosystem II (PSII) reaction centre (RCII) photodamage (Demmig-Adams et al., 2012). In addition, excessive reduced power favours over-production of reactive oxygen species (ROS), which are closely related to membrane damage (Shigeoka and Maruta, 2014), photoinhibition (Nishiyama et al., 2011) and even plant death (Foyer and Noctor, 2003).

Consequently, to plants survive under excessive light; evolution pressures have selected several photoprotective mechanisms (Ruban, 2015). These mechanisms are specifically associated to minimization of light energy input into photosystems and/or optimization of metabolic energy sinks (Foyer et al., 2012). Moreover, these responses are species-dependent and complexly regulated during excessive light response (Li et al., 2009a). If plants are able to

successfully activate defence mechanisms and sustain homeostasis, the organism becomes acclimated and, if the trait is stabilized throughout generations, the species is considered adapted (Souza and Lüttge, 2015). The current review is focused on main mechanisms related to photoprotection and photosynthetic homeostasis maintaining under excessive light conditions. Moreover, since none of these defence mechanisms act independently in a complex context such as plant cell, it will be given a special focus on integrative routes involving such mechanisms.

2. Photosynthesis and excess light

2.1. Photosynthesis, a general overview

Photosynthesis is the primordial metabolic process associated to life sustaining in planet Earth (Raven, 1984). By this process, photons are harvested by photosynthetic organisms and utilized to generate reducing power (reduced ferredoxin, NADPH and ATP). Subsequently, this energy is employed by different metabolic processes of plant cell, according to circumstantial demand (Blankenship, 2013). Among these processes, light-dependent CO_2 assimilation is believed to be the most important energetic sink in green tissues (Foyer et al., 2012; Silveira and Carvalho, 2016). However, in nature, light and CO_2 amounts greatly vary according to oscillations in environmental conditions. Thus, to succeed for millions of years, land plants needed to rise a very complex system related to photosynthesis optimization including both photochemical and CO_2 assimilation phases (Ruban, 2015).

A chain involving several protein-pigment super-complexes are the site of photochemical reactions in higher plants, which can be summarized in the following four key events:

1) Quantum energy from light photons are promptly harvested by antenna complexes;
2) This energy is employed to H_2O splitting and oxygen evolution at PSII reaction centre (RC);
3) Electrons obtained from water splitting are carried through an electron transport chain producing a proton gradient between lumen (acid) and stroma (basic) into chloroplasts. This gradient is a key event related to several other processes such as ATP synthesis.
4) At PSI, electrons are used in $NADP^+$ reduction, producing NADPH (Hill and Bendall, 1960).

The NADPH and ATP generated in photochemical activity of thylakoid membranes can be utilized as energy source for several different metabolic processes, such as CO_2 assimilation in Calvin cycle reactions. Indeed, CO_2 assimilation is believed to be the most important electron sink for PSII. Carbohydrates generated as consequence of Calvin cycle activity, such as sucrose, can be transported to different plant organs, which is crucial to sustain plant growth and development. Calvin cycle pathway involves the following three main events:

1) Carboxylation phase, which is the most primordial reaction related to CO_2 assimilation (considering C3 plants) and is catalysed by the most abundant protein in Earth planet, ribulose-1-5-bisphosphate carboxylase/oxygenase enzyme (Rubisco), which can use both CO_2 and O_2 as substrate;
2) Reduction phase. At this phase are produced phosphor-trioses, which are important precursors for carbohydrate synthesis. A great fraction of reducing power produced

by photochemical activity in thylakoid membranes is therefore utilized in this photosynthetic phase;

3) Regeneration phase, which encompasses regeneration of ribulose-1-5-bisphosphate, restarting the cycle (Calvin and Benson, 1948). This phase involves several enzymes such as sedoheptulose-bisphosphatase and fructose-bisphosphate aldolase, which are activated by light.

2.2. PSII-LHC super-complexes, the main core of higher plants photochemical activity

Photosynthesis is a very complex metabolic process, which involves multiple interconnected sub-processes. Evidently, to none of these sub-processes can be considered as more relevant in terms of promoting understanding of higher plants responses, which can lead to excessive light resistance (Silveira and Carvalho, 2016). Nevertheless, in order to maintain current study focus on integrated photoprotective mechanisms, which allow plants support photosynthesis under excess light, a deeper comprehension into PSII-LHC super-complexes structure is required. The PSII super-complex is a protein-pigment system present in thylakoid membranes of higher plants, at stacked grana sites, which are categorized in two distinct sub-complexes:

a) Light harvesting complex (LHC) PSII antennas, which is related to light energy capture and energy partition;
b) PSII core, which encompasses PSII reaction centers (RC) intrinsic proteins. These proteins are involved in oxygen evolving complex (OEC), where H_2O splitting is associated with O_2 evolution (Bricker et al., 2012; van Amerongen and Croce, 2013; Pagliano et al., 2013).

c) PSII extrinsic proteins, which are associated with OEC activity enhancement, despite been not indispensable for O_2 evolution Bricker et al., 2012).

Recent structural studies have reported PSII-antenna as organized into four (two strongly binding and two moderately binding) major antenna complexes (LHCII) (van Amerongen and Croce, 2013). LHCII, in turns, consist of one trimeric unit: Lhcb1, Lhcb2 and Lhcb3 proteins (Horton and Ruban, 2005; van Amerongen and Croce, 2013) and three monomeric proteins containing two independent monomers each (minor antennas), referred as CP29 (Lhcb4), CP26 (Lhcb5) and CP24 (Lhcb6) proteins (van Amerongen and Croce, 2013). It is well known that antenna proteins are capable to bind several pigment molecules, such as chlorophyll *a* and *b*, lutein (Lut), violaxanthin (Vx)/zeaxanthin (Zx) and neoxanthin (Nx) (Peter and Thornber, 1991).

These pigment-protein complexes are also related to non-photochemical quenching (NPQ) formation and excess energy dissipation as heat qE (Ruban et al., 2012). This process is strongly dependent on roles of Lhcb4 in LHC macro-structural organization (de Bianchi et al., 2011) and the presence of subunit S of PSII (PsbS) (Funk et al., 1995). PSII core complex is probably composed by about 20 subunits (Umena et al., 2011; van Amerongen and Croce, 2013). At least seven subunits are believed to be indispensable for water splitting and oxygen evolving process, including CP47 (PsbB), CP43 (PsbC), D1 (PsbA), and D2 proteins (PsbD). Additionally, α (PsbE) and β (PsbF) subunits of cytochrome b559 and 4.8 kDa *PsbI* gene product also belong to PSII core (Bricker et al., 2012; van Amerongen and Croce, 2013). A hypothetic PSII tri-dimensional structure can be visualized in figure 1.

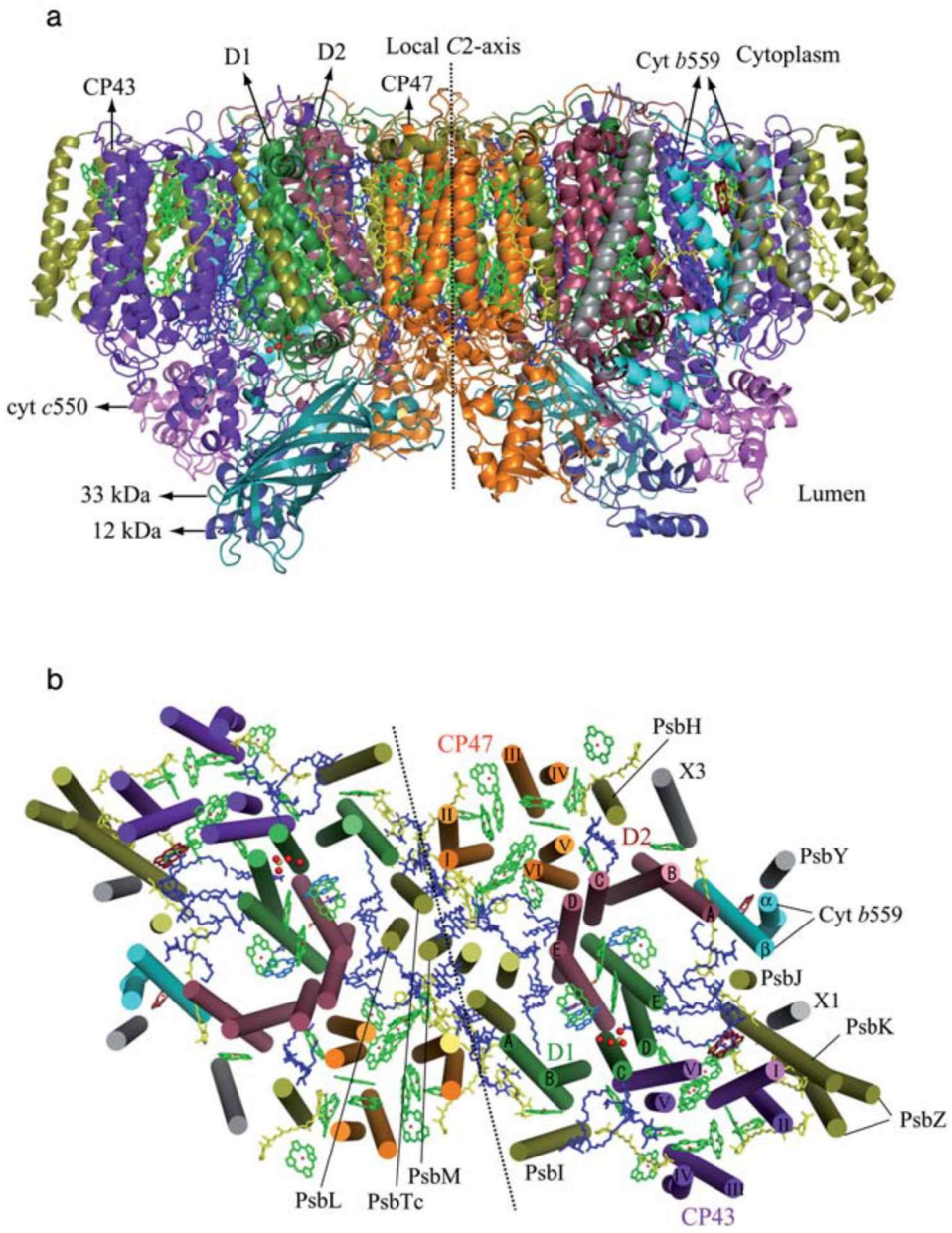

Figure 1. Crystal structure of PSII at 3.0 Å resolution from *Thermosynechococcus elongatus*. (a) Side view of PS II dimer along with the membrane plane. (b) The arrangement of TMHs and cofactors seen as a top view of PS II dimer perpendicular to the membrane plane from the lumenal side. Major protein subunits are coloured as follows: D1 (green), D2 (red), CP47 (orange), CP43 (purple), α - and β – subunits of Cyt b 559 (cyan), and the three extrinsic proteins 33 kDa (cyan), Cyt c 550 (pink), 12 kDa (deep blue). Cofactors are shown as: chls (green), carotenoids (yellow), lipids (deep blue), Q A and Q B, Pheo (blue), Mn, Fe and heam ring of Cyt (red), and Ca (yellow). Copied from Shen et al., (2008) - *Wiley-VCH Verlag GmbH & Co*.

2.3. Excessive light and photoinhibition

The majority of plants in nature are not exposed to optimal environmental conditions for growth (Goh et al., 2012). Indeed, for the most conditions high incident light (high input) or impairment of CO_2 assimilation (low output) and in other important metabolic processes can lead to photosynthetic unbalance, which is a serious threat for plants surviving (Ruban, 2015). This condition is defined as excess light stress (Fig. 2). Excess light stress may be particularly a relevant condition for tropical and equatorial regions, where radiation can reach very high levels between periods of cloud cover. Here, the average annual solar irradiation can exceed 1800 kWh/m^2, typical of dry seasons in the tropics (Murchie et al., 2015). Regions such as Central Luzon in Philippines and Central and East Java in Indonesia can receive accumulative annual solar irradiation of more than 2200 kWh/m^2 (Murchie et al., 2015). Moreover, combination of high light irradiance and drought, or salt stress, both usual conditions in semi-arid regions could severely aggravate excessive light stress in plants (Ferreira-Silva et al., 2011; Silva et al., 2015; Silveira and Carvalho, 2016).

Plants exposed to excess light accumulates more reducing power than circumstantial energetic demand (Li et al., 2009a; Takahashi and Badger, 2011; Goh et al., 2012). Under these conditions, several side-reactions associated to photosynthetic electron transport chain become more probable, such as reduction of O_2 at PSII (Zulfugarov et al., 2014) and PSI (Asada, 1999), producing ROS. Indeed, plant cells can produce high levels of ROS even under optimal growth conditions, but, in an excessive light state, ROS levels can over-accumulate (Foyer and Noctor, 2003; Goh et al., 2012; Shigeoka and Maruta, 2014). Moreover, over-accumulation of ROS in chloroplasts is one of the most important factors associated to photoinhibition (Takahashi and Badger, 2011; Murchie et al., 2015)

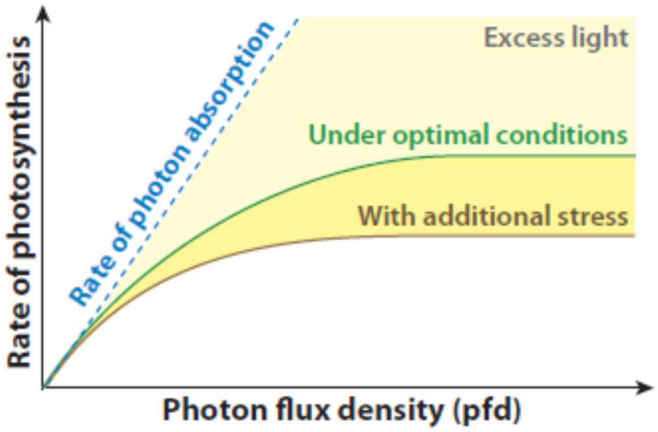

Figure 2. Light response curves for photosynthesis compared with the rate of light absorption. Figure copied from Li et al., (2009a) - *Annual Review of Plant Biology*.

Photoinhibition is the reduction of photosynthetic rate in response to conditions of prolonged or pronounced excess light absorption. Under excessive light conditions, double reduction of plastoquinone (PQ) pool ceases the electron transport, which inhibits PSII activity. In addition, charge recombination reactions occurring in the inhibited PSII are more likely to lead to triplet state of the primary donor (P680), favouring production of oxygen singlet (Aro et al., 1993). Photoinhibition of PSII occurs in consequence of the produced singlet oxygen as well as by weakly coupled chlorophyll molecules (Goh et al., 2012). It has been therefore considered that, even in a healthy leaf, oxygen evolving complex (OEC) does not always function in all PSII centres, and these RC are prone to a rapid and irreversible photoinhibition (Goh et al., 2012).

At PSI level, Mehler-reaction is capable to also reduces O_2 generating superoxide ions $O_2^{\bullet-}$ and in a subsequent reaction catalysed by superoxide dismutase (SOD), $O_2^{\bullet-}$ is rapidly converted in H_2O_2 (Mehler, 1951). Hydrogen peroxide is capable to easily transpose organelles membranes and defund to virtually all cellular compartments (Mubarakshina et al., 2010). Moreover, H_2O_2 is believed to be associated with several signalling routes involved in plant abiotic stress responses, modulating gene expression and enzymatic activities (Karpinski et al., 1997; Desikan et al., 2001; Mhamdi et al., 2012; Karpiński et al., 2013; Corpas, 2015). In

opposition, when H_2O_2 is over-accumulated in plant cells, this molecule is able to interfere in elongation factor G, affecting D1 *de novo* synthesis and generating impairment in RC repair mechanisms (Nishiyama et al., 2011). Figure 3 summarises ROS-dependent photoinhibitory mechanisms involving elongation factor G and PSII RC repair mechanisms.

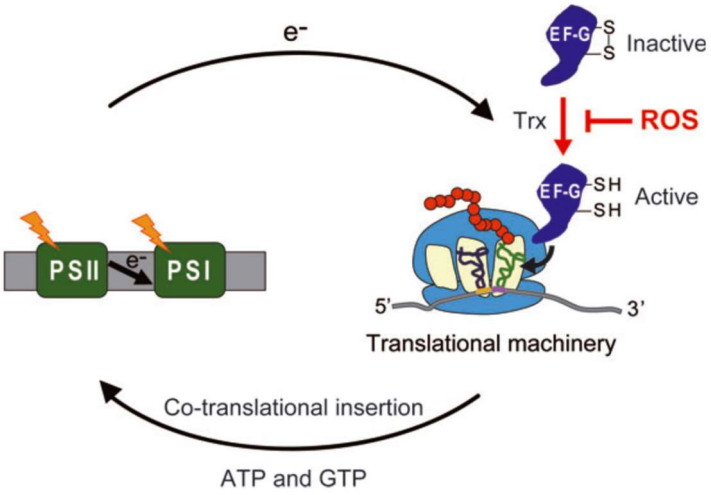

Figure 3. A schematic representation of the interactive regulation of photosynthesis and protein synthesis. In light, reducing equivalents that are generated by the photosynthetic transport of electrons (e−) are transmitted to EF-G via thioredoxin-dependent (Trx-dependent) redox pathways. The resultant reduction of EF-G activates the translational machinery, leading to induction of the synthesis of the D1 protein and other proteins. The repair of PSII, in turn, requires the synthesis de novo of proteins and, in particular, that of the D1 protein. High levels of ROS, produced by the photosynthetic machinery, interrupt the redox signals by maintaining EF-G in an oxidized state and, consequently, they suppress protein synthesis that is required for the repair of PSII. Copied from Nishiyama et al., (2011) - *Physiologia Plantarum*.

Consequently, production and accumulation of ROS is one of the main side effects of imbalance generated by excessive light. The high reactivity of ROS may lead to damages in thylakoid membranes, protein carboxylation and DNA damage (Takahashi and Badger, 2011; Shigeoka and Maruta, 2014). Thus, in order to survive at different environmental conditions related to excessive light, higher plants needed to develop several mechanisms of photoprotection (Takahashi and Badger, 2011). These mechanisms can be resumed into two main strategies: a) decreasing energy input, which encompass several mechanisms, such as leaf and chloroplast movements, PSII antennas-RC connectivity, reversible photoinhibition and

NPQ; b) increase of energy output, which involves stimulation of CO_2 assimilation, higher photorespiratory activity, triggering of antioxidant defences, induction of NO_3^+ assimilatory metabolism and activation of other reductant metabolic processes (Takahashi and Badger, 2011; Silveira and Carvalho, 2016). In figure 4, is possible to summarise the main mechanisms related to photoprotection in plants (Takahashi and Badger, 2011).

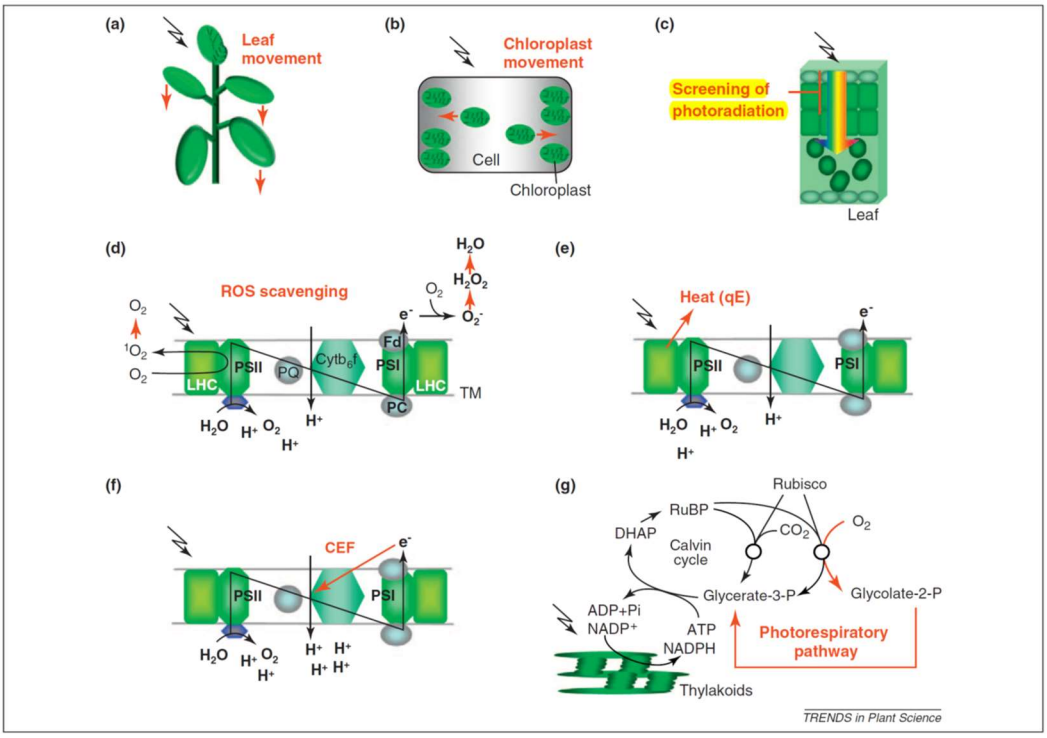

Figure 4. Examples of leaf and chloroplast mechanisms involved in minimizing photoinhibition of PSII. (a) Leaf movement. Leaves move to minimize the absorption of excessive light. (b) Chloroplast movement. Chloroplasts change their position to minimize the absorption of light. (c) Screening of photoradiation, for example, UV screening by phenolic compounds in epidermis cells. (d) ROS scavenging. O_2 produced at PSII is scavenged by membrane-bound a-tocopherol and carotenoids. O_2^- and H_2O_2 produced at PSI are scavenged enzymatically and non-enzymatically by ascorbate. (e) Thermal energy dissipation of absorbed light energy (qE). qE dissipates light energy absorbed by photosynthetic pigments as heat at minor light-harvesting proteins. (f) CEF around PSI. CEF includes both the NAD(P)H dehydrogenase complex-dependent and PGR5-dependent pathways and helps to generates ∆pH across the thylakoid membrane. (g) Photorespiratory pathway. Glycolate-2-P generated by the oxygenase reaction of Rubisco is recycled into the Calvin cycle intermediate glycerate-3-P through the photorespiratory pathway. Reproduced from Takahashi and Badger (2011) - *Trends in Plant Science*.

3. Photoprotective mechanisms

In order to survive under different light conditions as previously mentioned, natural selection pressures have induced the development of several different strategies to optimization of light capture and energy use in plants (Ruban, 2015). Some of these strategies are related to maximization of light capture, which include emergence of antenna complex structures, increases in proportion of antennas/RC ratios and optimization of light quality use by processes such as PSII/PSI state transitions (Puthiyaveetil et al., 2012). These mechanisms are generally associated to shadowing and low light conditions and will not be focused on current review. In contrast, under excessive light, a very usual situation, several other mechanisms related to minimization of over-accumulated energy are described in literature (Takahashi and Badger, 2011; Goh et al., 2012). These mechanisms are now focus of the next section.

3.1. Leaf position and light incidence

Position of leaves related to predominant incidence angle of light is a very important phenomenon and have been target of several studies during the last five decades (Kriedeman et al., 1964; Murchie et al., 1999; Fourcaud et al., 2008; Baccar et al., 2011; Burgess et al., 2015). Indeed, two main factors are primordial for photosynthetic activity of individual leaves: disposition of leaves in relation to the incidence of light and the inherent optical proprieties of leaves (Kriedeman et al., 1964). Individual leaf photosynthetic light intensity is proportional to cos θ, where θ is equivalent to the light incidence angle (Kriedeman et al., 1964). In addition, θ can deeply vary in according to species and even inside the canopy of a same individual (Murchie et al., 1999).

Plant canopy are complex three-dimensional objects with a great variability in leaf size, shape, area, angle, curvature, twisting, and clumping (Burgess et al., 2015). These properties are especially important for studies involving *gramineae* plants, such as rice and wheat (Kriedeman et al., 1964; Murchie et al., 1999; Fourcaud et al., 2008). In these plant species, young leaves can exhibit 90° on light incidence angle and, under such conditions, cos θ equals to zero. Thus, differences in leaves positioning can represent a great strategy of photoprotection for several plant species, especially at physiological phases where leaves are particularly less light-tolerant (Carvalho et al., 2015). Interestingly, several plant species evolved complex physiological mechanisms associated to leaf movements, which, in turns can actively modulates θ angle of light incidence, and protect photosystems from photoinhibition under excessive energy (Takahashi and Badger, 2011).

Heliotropism, the leaf movement related to sunlight angle of incidence, consists in two distinct movements: diaheliotropism (leaf blade becomes oriented perpendicular to the angle of light incidence) and paraheliotropism (leaf blade becomes oriented perpendicular to light incidence angle). Especially, paraheliotropism is associated with minimizing energy capture, consisting in a photoprotective mechanism (Kao and Forseth, 1992; Pastenes et al., 2005). Paraheliotropism movements can reduces photoinhibition under excessive light conditions by two main strategies:

a) Reducing light input into antennas super-complexes by alterations in θ angle;
b) Reducing the probability of ROS generation, because of lower energy accumulated in thylakoid membranes, and therefore avoiding detrimental effects of ROS in PSII repair mechanisms (Takahashi and Badger, 2011).

In addition to leaf movements, chloroplast movements into mesophyll cells are also believed to act as important photoprotective mechanisms in plants under excessive light

conditions (Takahashi and Badger, 2011). In *A. thaliana* plants, two proteins have been characterized as photoreceptors phototropin 1 (PHOT1) and phototropin 2 (PHOT2) and as essential to chloroplast movement (Sakai et al., 2001; Kasahara et al., 2002; Oikawa et al., 2003). The mechanistic basis of leaf chloroplast movements are actin filaments and chloroplast outer envelope protein CHUP1 is believed to be very important for this photoprotective mechanism (Takahashi and Badger, 2011).

3.2. Modulation of LHC-PSII genes/proteins and chlorophyll content under excessive light

Plants have evolved several different mechanisms to avoid excessive input of light energy into photosynthetic electron carriers. These mechanisms include active modulation in the organization and/or abundance of protein complexes in thylakoid membranes (Timperio et al., 2012), decreased levels of major chlorophyll a/b-binding light-harvesting complexes in relation to of reaction centers (Borisova-Mubarakshina et al., 2015), higher chl *a*/*b* ratios (Bailey et al., 2001; Ballottari et al., 2007), and modulation of PSII/PSI ratios (Puthiyaveetil et al., 2012; Borisova-Mubarakshina et al., 2015). Nevertheless, Zivcak et al., 2014 working with barley leaves concluded that decrease of chl *a*/*b* ratios in low light conditions strongly depends on plant species (Zivcak et al., 2014). Moreover, the authors evoked the theory of PSII connectivity to explain obtained data. According to this theory, PSII RC's are interconnected and excitation energy should be distributed among then under illumination (Kramer et al., 2004). If it is true, the sigmoidal shape of fluorescence induction could affect the efficiency of utilization of absorbed light for trapping electrons in RC and hence, it should reflect on the entire fluorescence kinetics (Zivcak et al., 2014).

Despite these interesting results, it was widely reported excessive light as capable to modulate several nuclear genes associated to photosynthesis (Murchie et al., 2005; Li et al., 2009a). Several regulation mechanisms are related to control the synthesis of chlorophyll by

regulation of various intermediates of tetrapyrrole synthesis, such as 5-aminolevulinic acid - ALA (Li et al., 2009a). Moreover, increased Mg-Proto levels in plants exposed to photooxidative stress have been reported in the literature (Strand et al., 2003; Strand, 2004). In addition, ROS levels are believed to be related to accumulation of Mg-ProtoMe (Aarti et al., 2006). Figure 5 shows some of the most important routes of chloroplast signalling responses related to excessive light.

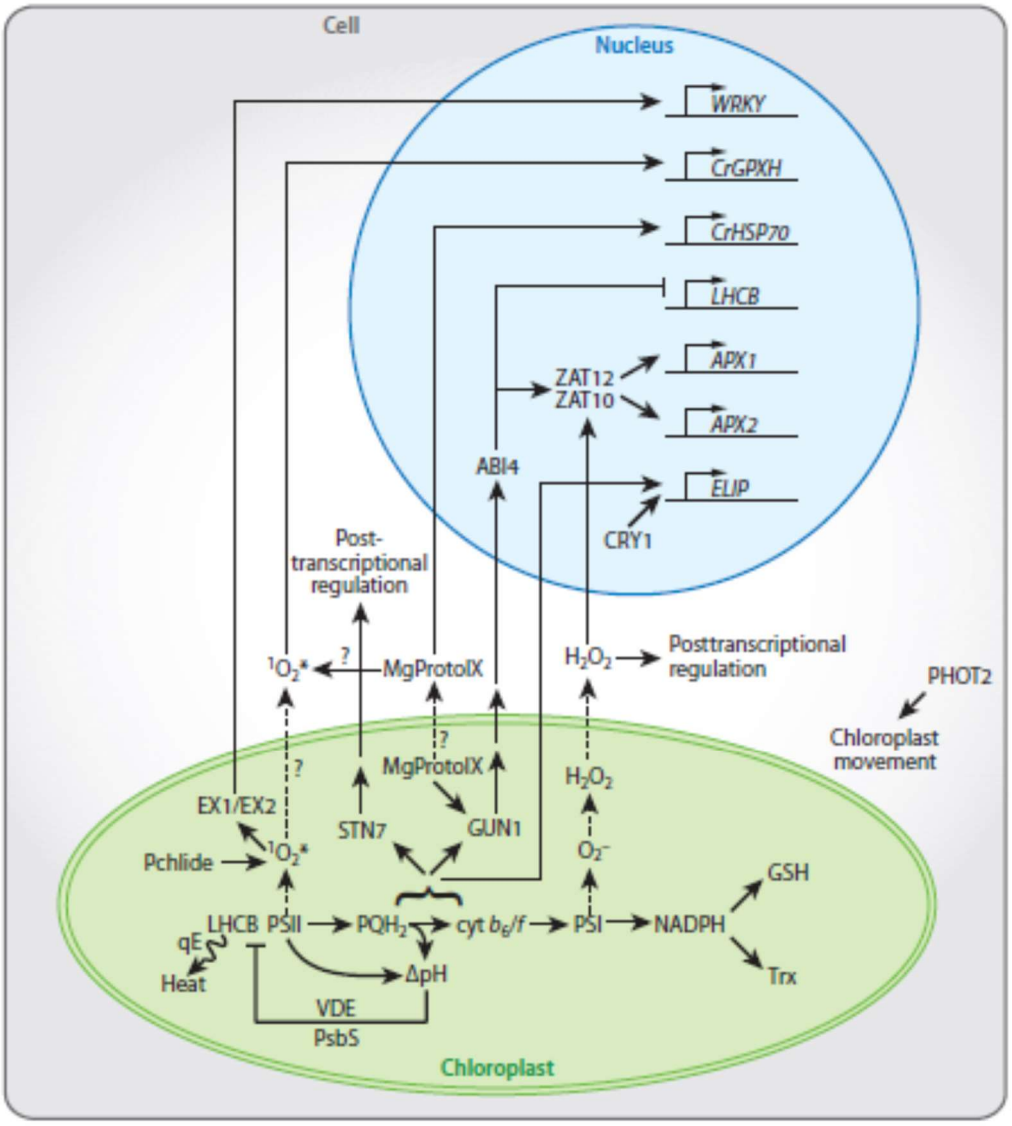

Figure 5. Schematic diagram of a plant/algal cell showing the locations and interactions of some of the sensing and signalling molecules that are involved in responses to excess light. Dashed arrows indicate diffusion or transport of a molecule. cyt b6/f, cytochrome b6/f complex; GSH, glutathione; Mg-ProtoIX, Mg-protoporphyrin IX; qE, pH-dependent regulation of photosynthetic light harvesting; PS, photosystem; PQH2, plastoquinol; PChlide, protochlorophyllide; Trx, thioredoxin; VDE, violaxanthin de-epoxidase. Reproduced from Li et al., (2009) - *Annual Review of Plant Biology*.

Regulation of LHCB genes expression have recently been related to H_2O_2-dependent signalling pathways (Borisova-Mubarakshina et al., 2015). Indeed, H_2O_2 roles in signalling mechanisms are widely reported in literature (Desikan et al., 2001; Ślesak et al., 2007; Karpiński et al., 2013). Accordingly, results reported by Borisova-Mubarakshina et al., (2015) support the hypothesis that H_2O_2 is a molecular signal, which contributes to regulation of PSII antenna size under excessive light, a response directly related to long-term high light acclimatory performance. In summary, the authors proposed H_2O_2 molecule released by PQ-pool (Zulfugarov et al., 2014), as one of the main candidates by which redox state of PQ-pool provides antennas size regulatory effects (Figure 6).

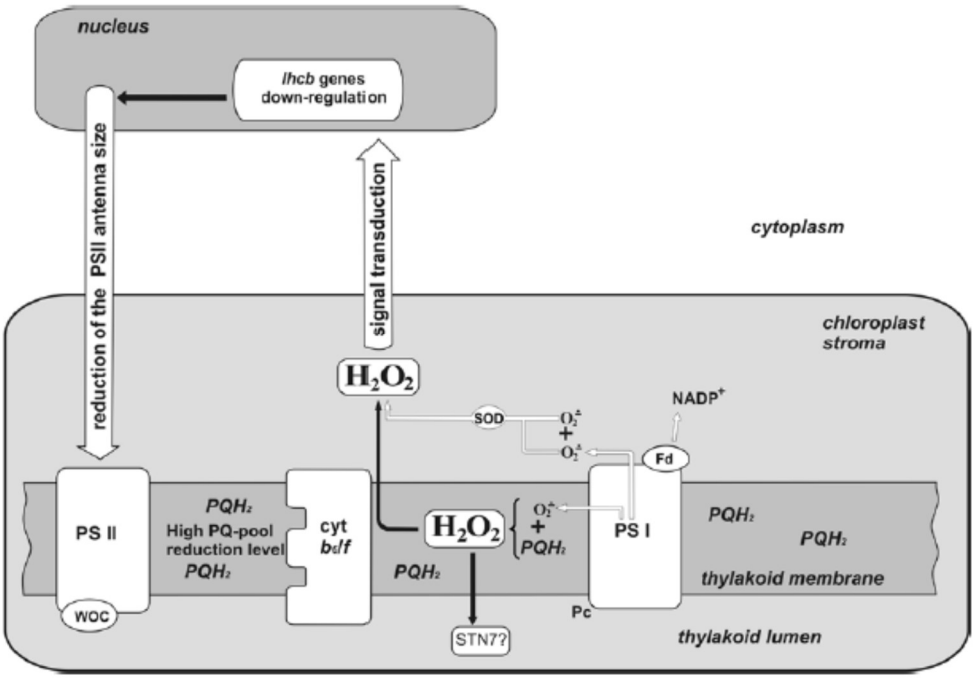

Figure 6. A tentative scheme of H_2O_2 production in chloroplasts and a possible signal transduction pathway for the down-regulation of *lhcb* genes by H_2O_2 originated in the PQ pool, involved in the reduction of PSII antenna size under HL conditions. White arrows, superoxide radical and hydrogen peroxide production in chloroplast; Fd, ferredoxin; PSII, photosystem II; PSI photosystem I; PQ, $PQ^{\cdot -}$, PQH_2, plastoquinone, plastosemiquinone, plastohydroquinone respectively; Pc, plastocyanin; SOD, superoxide dismutase; WOC, water-oxidizing complex. The PQH_2/PQ ratio rises under long-term HL conditions; PQH_2 spreads along the entire thylakoid membrane thus increasing the probability of reaching a superoxide radical, and to produce hydrogen peroxide within the membrane. Reproduced from Borisova-Mubarakshina et al., (2015) - *Journal of Experimental Botany*.

Fabricio E.L. Carvalho

3.3. NPQ – dissipating excess energy as heat

Natural selection pressures have conducted the development of several molecular mechanisms in plants related to excessive light tolerance (Goss and Lepetit, 2014). These molecular mechanisms are classified into two distinct groups of responses: long-term acclimation and short-term regulatory responses (Ruban et al., 2012). Among short-term acclimatory responses, an additional control loop in the form of a proton effect upon the PSII energy conversion event have been considered a crucial mechanism for plant defence against excessive light (Briantais et al., 1979; Genty et al., 1989). This process can be monitored by chlorophyll *a* fluorescence yield declining under conditions of photochemical excitation energy accumulation and is called non-photochemical quenching (NPQ) - (Govindjee et al., 1966; Murata and Sugahara, 1969; Wraight and Crofts 1970; Ruban, 2016). The figure 7 displays a synthetic model for NPQ activation in higher plants.

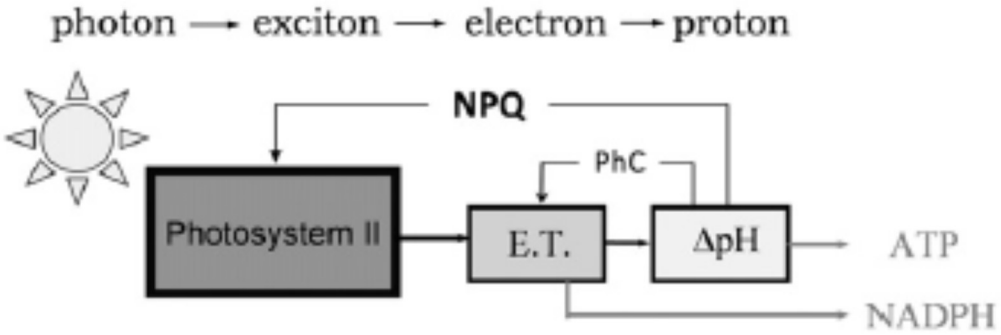

Figure 7. Scheme depicting photosynthetic and feedback control of photosynthesis in higher plants. PSII absorbs light and produces electrons (E.T.) which are transported through the thylakoid membrane to produce NADPH and drive formation of ∆pH for ATP synthesis. Build up ∆pH exerts control over photosynthesis via inhibition of electron transport (photosynthetic control, PhC) and via regulation of the excitation pressure in PSII (NPQ). Reproduced from Ruban et al., (2012) - *Biochimica et Biophysica Acta*.

NPQ is a complex mechanism composed of at least four major processes known to date: state transition (qT), photoinhibition (qI), heat dissipation (qE) and zeaxanthin-dependent (qZ) quenching (Nilkens et al., 2010; Ruban, 2016). These processes have individual features and probably reflect very distinct metabolic routes. Redistribution of light energy capture between PSII and PSI is believed to be related with qT (Benson et al., 2015). This process involves a partial detachment and migration of LHCII between PSII and PSI according to light conditions and is believed to be very important in algae (Baker, 2008; Puthiyaveetil et al., 2012). In higher plants, nevertheless, qT protective roles under excessive light have been questioned and qT functioning have been more associated to low light conditions (Baker, 2008).

The qI component is a slowly reversible process, which probably reflects damage to RCII and photoinhibition (Ruban et al., 2012). Photoinhibition by itself process can be considered as a protective mechanism in terms of whole plant surviving, since closed RCII confers protection compared to open ones (Matsubara and Chow, 2004). However, the strict threshold, which photoinhibition can be considered as a defence mechanism or, contrastingly represents an excessive light stress symptom is a hard to define. Indeed, classification of stress symptoms against defence responses is not a simple task (Silveira and Carvalho, 2016).

Two components are believed to act as direct protective mechanisms for avoiding damages to RCII, and therefore, photoprotection under excessive light. The fastest component of NPQ, and also the more frequently studied in plants, is named qE, and its photoprotective role is probably related to dissipation of excess energy as heat (Ruban et al., 1993). More recently described, qZ is probably related to synthesis and epoxidation of zeaxanthin and probably display roles in photoprotection. The lifetime of qE and qZ formation/relaxation present order of 5 and 15 minutes respectively, therefore consisting in the faster NPQ components (Nilkens et al., 2010; Ruban, 2016).

Mechanisms underlying qE formation are relatively well stablished in literature. First, increase of pH gradient between lumen (acid) and stroma (basic) in illuminated chloroplasts

because of photosynthetic electron transport in thylakoid membranes is the primordial event triggering qE (Krause, 1974; Ruban et al., 1992). Subsequently, zeaxanthin binding to light harvesting complexes is related to a new state of aggregation, which allows this system to work as a thermal dissipater of energy (Demmig-Adams and Adams, 1992; Ruban et al., 1992; Farber et al., 1997; Ruban et al., 1999). Finally, an important transmembrane chl *a*-*b* binding protein with 22 kDa was discovered to display a crucial role in qE activation (Funk et al., 1995; Li et al., 2002; Niyogi et al., 2005). This protein called PsbS, presents a particular role of increase sensitivity of thylakoid membranes to pH changes, allowing qE formation under physiological ∆pH values (Johnson and Ruban, 2011). Figure 8 and 9 summarises the mechanisms underlying qE formation in higher plants.

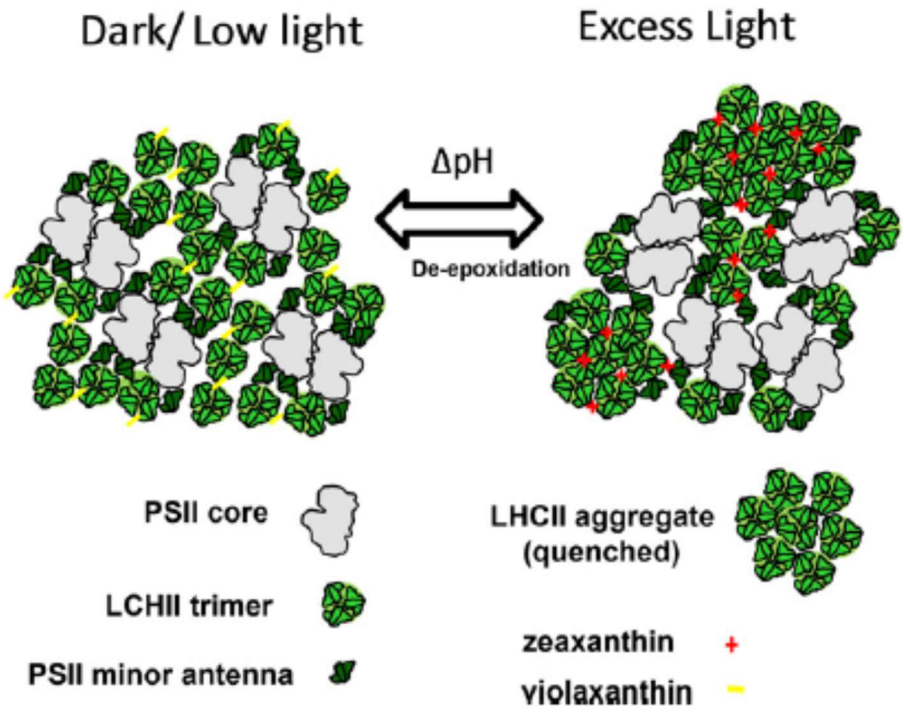

Figure 8. Structural model of NPQ-related reorganization of thylakoid grana membranes. In the dark and low light, LHCII is fairly distributed in the grana, forming large $C_2S_2M_2$ super-complexes with PSII and minor antenna proteins. In excess light, ∆pH triggers a conformational change within LHC complexes that causes the partial dissociation of the PSII-LHCII super-complex and leads to LHCII aggregation. De-epoxidation of violaxanthin to zeaxanthin promotes LHCII aggregation and, therefore, NPQ. Reproduced from Johnson et al., (2011) - *Journal of Biological Chemistry*.

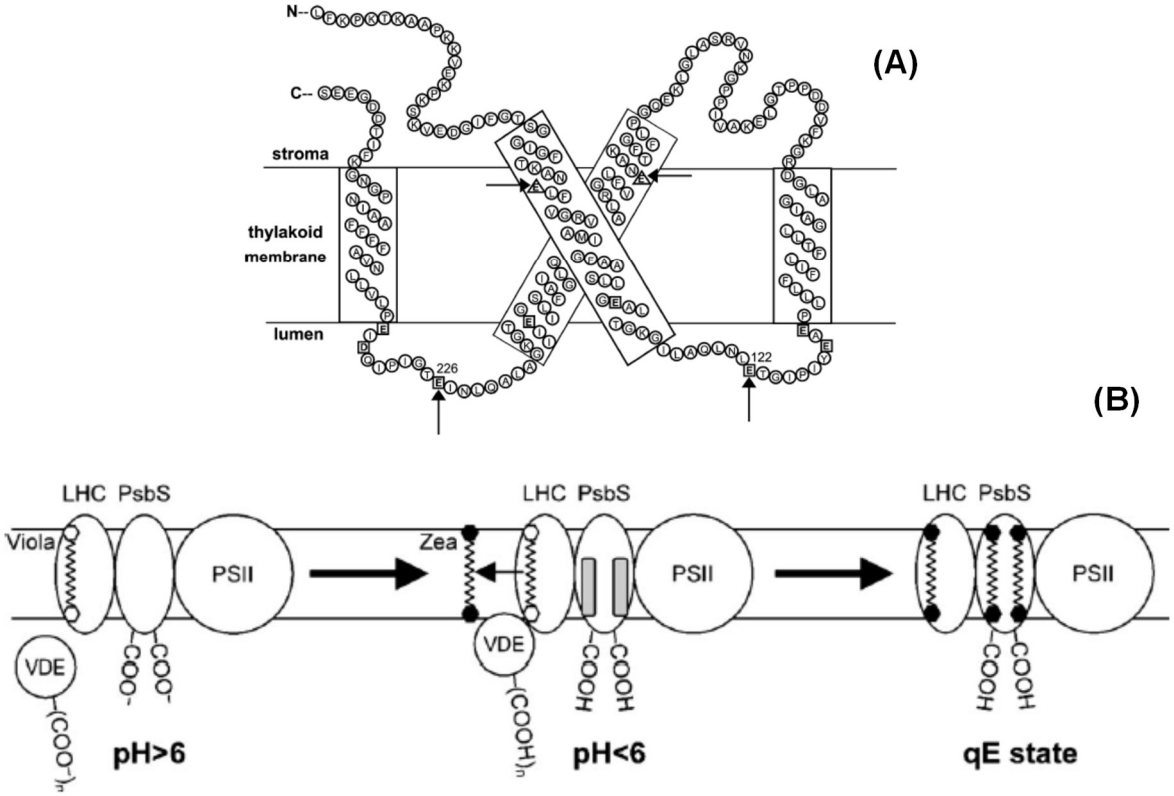

Figure 9. (A)Topological model of *A. thaliana* PsbS. Triangles and horizontal arrows denote positions of two highly conserved, charge-compensated glutamates that serve as ligands to bound chlorophylls in LHCII. Squares denote the positions of eight acidic amino acid residues (seven glutamates and one aspartate in Arabidopsis) located at or near the lumen side of the protein that are conserved in all known PsbS sequences. The two glutamates that are necessary for qE and DCCD binding are numbered and marked by vertical arrows. Numbering is relative to the predicted initiator methionine of the PsbS precursor protein (prior to import into chloroplasts). (B) Schematic model for qE in plants. (Left) At limiting light, the steady-state thylakoid lumen pH is greater than 6. Violaxanthin (Viola) is bound mainly to the V1 site in LHCII and the L2 site in other LHC proteins (such as CP29 and CP26). (Middle) In excess light, the thylakoid lumen pH drops below 6, driving protonation of carboxylate side chains in VDE and PsbS. Protonation of VDE activates the enzyme and allows for its association with the membrane, where it converts multiple Viola molecules to zeaxanthin (Zea). Protonation of glutamate residues E122 and E226 in PsbS activates symmetrical binding sites for xanthophylls with a deepoxidized b-ring end-group (i.e. zeaxanthin). (Right) Zea binding to protonated sites in PsbS results in the qE state in which singlet chlorophyll de-excitation is facilitated. Adapted from Niyogi et al., (2005) - *Journal of Experimental Botany*.

Nevertheless, the precise site of qE formation still have been theme of intense discussion in the last decades (Ruban et al., 1992; Ruban et al., 2007; Ruban et al., 2012; Chmeliov et al., 2015). Presently, there are three more discussed theories for explaining the site of qE formation, which all of them predicting for LHC antennas involvement:

1. Energy transfer by interactions between chlorophyll molecules bound to antenna protein complexes (Horton et al., 1996);
2. Quenching interactions between lutein bound at the L1 site and chlorophylls a612–a611–a610 of LHC antennas (Ruban et al., 2007);
3. Xanthophyll–chlorophyll quenching interactions between zeaxanthin bound at the L2 site and chlorophylls A5 and B5 of LHC complexes (Ahn et al., 2008).

In order to determine the precise region of LHC antennas related to qE formation, several studies involving genetic transformation approaches have been performed. Absence of CP26 subunit did not affected qE, while CP29 and CP24 deficiencies promoted 30% and 50% respectively of qE impairment (Andersson et al., 2001; Kovács et al., 2006). Intriguingly, double deletion for CP29 and CP24 combined with 50% reduction in CP26 levels did not affected qE (de Bianchi et al., 2008). In parallel, deficiency in Lhcb1 and Lhcb2 (major antennas), lead to 35% decrease in qE (Andersson et al., 2003). Accordingly, reports on plants containing only CP26 exhibited only a partial reduction in qE (Jahns and Krause, 1994). Therefore, it is plausible to suggest no individual LHCII complex acting as the sole site of qE and, in principle, the quenching could occur in any of the four types of LHCII (Ruban et al., 2012).

Regardless qE generation specific site, it has been evidenced in several studies, especially involving PsbS deficient plants (Graßes et al., 2002; Ware et al., 2014) and violaxanthin de-epoxidase impaired mutants (Havaux et al., 2000; Niyogi et al., 2001) that plants deficient in qE formation are more susceptible to light stress. Nevertheless, is very important to highlight that a quantitative relationship between NPQ parameter (Fm/Fm′–1), and

actual photoprotection of PSII has not been demonstrated yet (Jahns and Holzwarth, 2012). Therefore, in order to understand the physiological importance of different NPQ processes and their photoprotection effects, future work will have to explore this relationship in detail. A new fluorimetric methodology for photoinhibition tracking, based on qPd parameter quantification have been introduced recently (Ruban and Murchie, 2012).

The qPd approach presents a high potential to investigations involving NPQ and photoprotection since it allows to estimate the precise NPQ fraction associated with photoprotection, pNPQ (Ruban and Belgio, 2014; Ware et al., 2014; Carvalho et al., 2015; Giovagnetti and Ruban, 2015). This methodology has been successfully employed so far and positive interactions have been demonstrated between pNPQ parameter and PsbS protein expression (Ware et al., 2014), oxygen evolution complex activity (Giovagnetti and Ruban, 2015) and ontogenesis (Carvalho et al., 2015). Moreover, evidences have connected pNPQ and photoprotection to hydrogen peroxide and oxidative stress, however further studies are needed in that direction to clarify this question (Carvalho et al., 2015).

3.4. Antioxidative metabolism and photoprotection

Plants exposed to excessive light, generally, display an over-accumulation of reduction power (Li et al., 2009a). Usually, this excess of reducing power is produced because of unbalance between NADPH and ATP production in photosynthetic electron transport chain (PETC) and energetic demand of general cell metabolism (Foyer et al., 2012). Under such conditions, formation of reactive oxygen species (ROS) is extremely favoured. The two most important sites of ROS generation in green leaf cells are peroxisomes and chloroplasts, followed by mitochondria (Foyer and Noctor, 2003). In peroxisomes, ROS are produced especially by activity of glycolate oxidase (GO), which scavenges glycolate, producing H_2O_2 (Corpas, 2015). This reaction is part of C2 cycle, involving recovery of carbon skeleton from phosphor-

glycolate produced by Rubisco oxygenation activity, also called photorespiration (Voss et al., 2013).

The most of cellular H_2O_2 produced is related to photorespiratory pathway in peroxisomes (Corpas, 2015). Indeed, peroxisomes of illuminated leaves are believed to generate up to 10 μmol m^{-2} s^{-1} of H_2O_2 (Foyer and Noctor, 2003). Several studies have reported H_2O_2 as important signalling molecules involved in abiotic stress responses (Karpinski et al., 1997; Desikan et al., 2001; Ślesak et al., 2007; Li et al., 2009a; Suzuki et al., 2013). However, H_2O_2 over-accumulation is involved in several harmful processes, such as protein carbonylation and lipid peroxidation, which are related to oxidative stress (Davletova et al., 2005). In peroxisomes, catalase (CAT) is a very important enzyme associated to H_2O_2 scavenging, independent of electron donors and presenting a high kM to hydrogen peroxide (Mhamdi et al., 2010).

Chloroplasts are the second most important site of H_2O_2 generation in illuminated leaf cells, reaching ratios up to 4 μmol m^{-2} s^{-1} (Foyer and Noctor, 2003). In chloroplast H_2O_2 is generated by different routes from that evidenced in peroxisomes, which involves other ROS such as radical ion superoxide ($O_2^{\bullet-}$) - (Mubarakshina and Ivanov, 2010). Indeed, two distinct theories have been used to explain H_2O_2 generation in chloroplasts (Asada, 1999; Mubarakshina and Ivanov, 2010; Zulfugarov et al., 2014). Earlier, H_2O_2 production was credited to PSI site, as a consequence of Mehler reactions (Mehler, 1951). In the PETC, electrons are carried to PSI Fe-S clusters and then to ferredoxin (Hill and Bendall, 1960). As O_2 is a very abundant molecule in chloroplasts, this molecule could take place of ferredoxin at PSI and act as electron receptor in the Hill chain (Mehler, 1951; Asada, 1999). The product of such reaction, $O_2^{\bullet-}$, is very reactive and harmful, and rapidly scavenged by superoxide dismutase activity (SOD), generating H_2O_2 (Asada, 1999).

However, a second theory supports H_2O_2 as being generated at PSII, instead PSI (Pospísil, 2009; Pospíšil, 2014; Zulfugarov et al., 2014). Indeed, the midpoint of redox potential

on electron acceptor side of PSI is more negative as compared with PSII, therefore O_2 reduction in PSI should occurs with high efficiency (Asada, 1999). However, evidences of possible involvement of PSII acceptor side in molecular oxygen reduction to superoxide have been recently reported (Pospísil, 2009; Pospíšil, 2014; Zulfugarov et al., 2014). This reaction should occur when semiquinone (QA^-) reduces O_2 generating superoxide, and subsequently, H_2O_2 (Fig. 10).

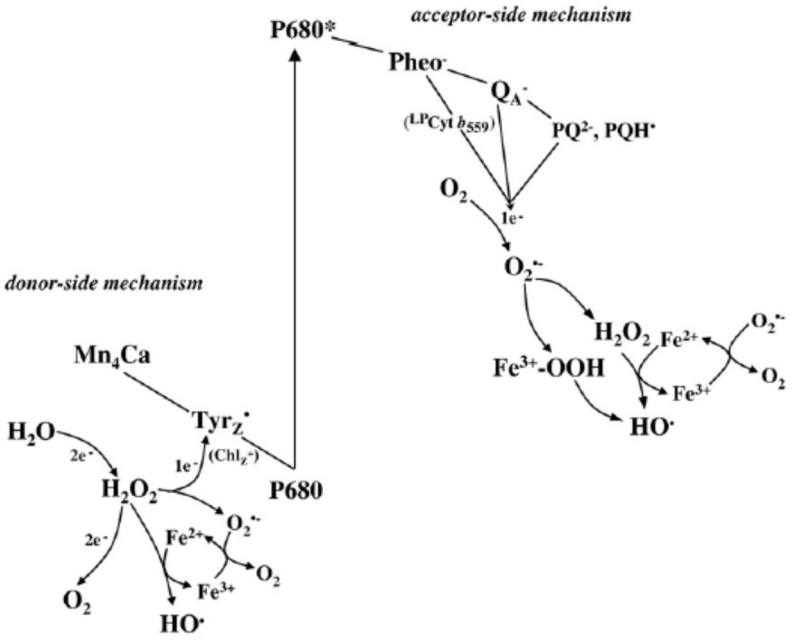

Figure 10. Production of ROS by acceptor- and donor-side mechanism in PSII. On the electron acceptor side of PSII, one-electron reduction of molecular oxygen by the reducing side of PSII results in the formation of $O_2^{\bullet-}$, which subsequently either dismutate it into free H_2O_2. In the presence of free metals (Fe^{2+}, Mn^{2+}), the reduction of H_2O_2 results in the formation of $HO^{\bullet}$ via Fenton-type reaction. On the electron donor side of PSII, two-electron oxidation of H2O by the modified water-splitting Mn complex results in the formation of H_2O_2. Hydrogen peroxide has been proposed to be either oxidized to $O_2^{\bullet-}$ by tyrosine radical $TyrZ^{\bullet}$ or reduced to $HO^{\bullet}$ by free metals the most likely being Fe^{2+}, Mn^{2+}released from damaged PSII. Reproduced from Pospíšil (2009) - *Biochimica et Biophysica Acta*.

In chloroplasts, CAT is absent, and therefore, ascorbate peroxidase (APX) was classically believed to be the most important enzyme involved in H_2O_2 scavenging (Nakano and Asada, 1981). Indeed, APX is conserved in most of higher plant species and is present in

almost all cell compartments (Teixeira et al., 2004). APX are encoded by multi-genic families and isoforms are classified according to different subcellular localization (Teixeira et al., 2004). In *Arabidopsis thaliana* L., there are at least six genes encoding APX isoforms: two cytosolic isoforms (At1g07890 and At3g09640); two peroxisomal isoforms (At4g35000 and At4g35970); a thylakoid-bound isoform (At1g77490) and a stromal/mitochondrial isoform (At4g08390) (Teixeira et al., 2006). In contrast, rice present 8 different APX isoforms: two cytosolic isoforms (*OsAPX1* and *OsAPX2*), two peroxisomal isoforms (*OsAPX3* and *OsAPX4*), one mitochondrial/chloroplastic isoform (*OsAPX5*), one mitochondrial (*OsAPX6*) and two chloroplastic, one stromal (*OsAPX7*) and one thylakoidal isoform (*OsAPX8*) - (Bonifacio et al., 2011).

Regardless cell compartment, APX activity is dependent of ascorbate (ASC) as electron donor for H_2O_2 scavenging reaction (Nakano and Asada, 1981). Ascorbate is the most abundant soluble antioxidant in chloroplasts, reaching concentrations up to 300 mM in these organelles (Smirnoff, 2000). ASC is synthetized in mitochondria and must be imported to chloroplasts in order to act as photoprotective molecule (Smirnoff, 2000). After ASC reach chloroplasts, this molecule can act in several mechanisms of photoprotection, such as:

1. Electron donor for APX activity;
2. Direct scavenging of other ROS;
3. Regeneration of α-tocopheryl radicals produced when α-tocopherol reduces lipid peroxyl radicals;
4. Alternative electron donor to PSII in absence of OEC function;
5. Substrate of violaxanthin de-epoxidase (VDE), crucial for qE.

These mechanisms mentioned above require the oxidation of ASC molecule, which generates monodehydroascorbate (MDHA) and dehydroascorbate (DHA). These molecules are

rapidly recovered by MDHA reductase or DHA reductase enzymes (DHAR) - (Foyer and Noctor, 2011). This last enzyme employs reducing power from reduced glutathione (GSH) for its activity, which, in turns, is regenerated from its oxidized form (GSSG) by activity of glutathione reductase (GR), using NADPH as electron donor (Foyer and Noctor, 2011). This cyclic process is referred as ascorbate-glutathione cycle and is considered a very important mechanism for ROS scavenging in chloroplasts under excessive light (Munné-bosch et al., 2013). Figure 11 summarises redox metabolic process in different plant cell organelles.

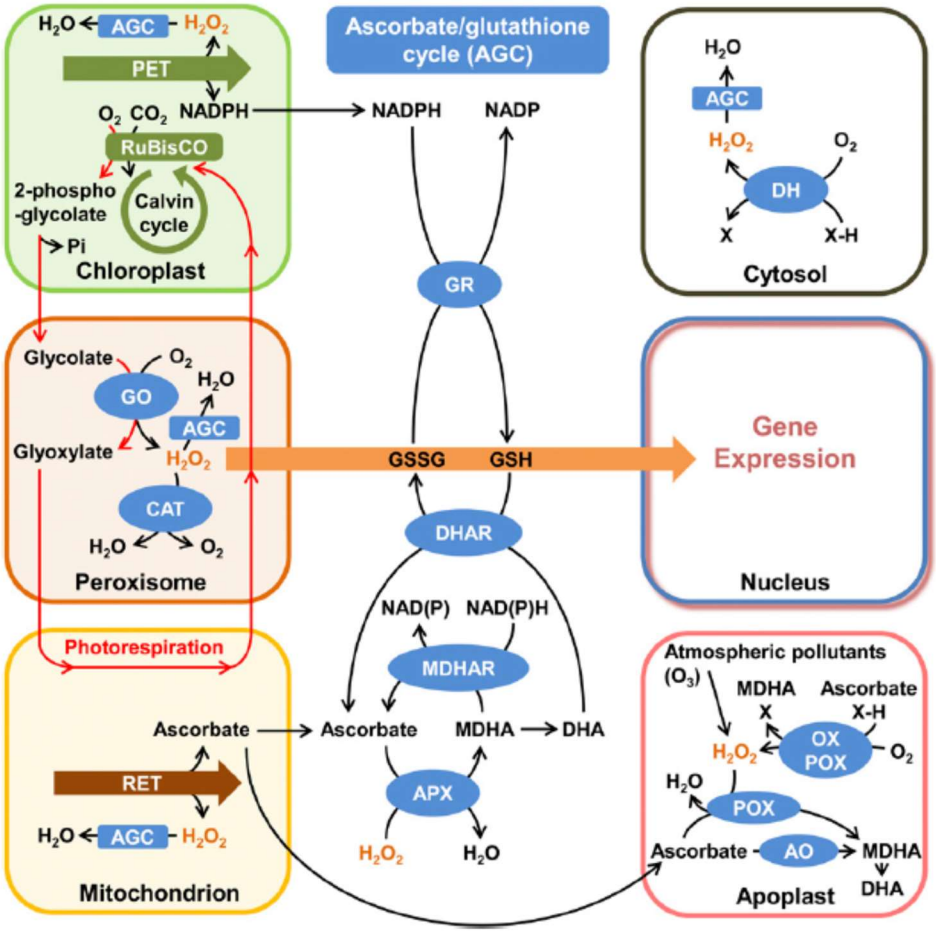

Figure 11. Schematic representation of subcellular hydrogen peroxide (H_2O_2) metabolism and its generation by the pathway of photorespiration and subsequent signalling through the glutathione pool linked to the ascorbate/glutathione cycle, as well as the production of ascorbate in the mitochondria and its subsequent transport to the apoplast, where it is oxidized by apoplastic enzymes related to extracellular redox signalling pathways. Reproduced from Munné-bosch et al., (2013) - *Plant Physiology*.

3.4.1. Does chloroplastic APX really play crucial role in plant responses to excessive light?

Plants exposed to excessive light display a strong induction of different isoforms of APX, especially cytosolic (Karpinski et al., 1997) and chloroplastic (Klein et al., 2012). Moreover, plants genetically transformed to over-express APX isoforms display increased tolerance to several abiotic stress conditions, for instance high salinity (Badawi et al., 2004; Li et al., 2009b; Singh et al., 2014). Based on these data, the answer to the question that intitules this section should be obvious. However, several studies employing the use of knocked out plants in order to assess the importance of these enzymes to abiotic stress responses have reported contrasting results (Danna et al., 2003; Giacomelli et al., 2007; Kangasjärvi et al., 2008; Maruta et al., 2010). Surprisingly, the majority of these works have reported no differences in transformed plants deficient in chloroplastic APX, in comparison to NT, under optimal growth conditions, and, sometimes even under abiotic stress (Danna et al., 2003; Giacomelli et al., 2007; Kangasjärvi et al., 2008; Maruta et al., 2010).

Excepting for wheat (Danna et al., 2003), deficiency in chloroplastic APX isoforms have produced plants capable to tolerate excessive light as well as NT (Giacomelli et al., 2007; Kangasjärvi et al., 2008; Maruta et al., 2010; Caverzan et al., 2014). Furthermore, it was discovered *A. thaliana* plants deficient in thylakoidal APX as displaying higher susceptibility to excessive light stress only in the simultaneous deficiency in ASC (Giacomelli et al., 2007) and in the first hours of high light exposure (Maruta et al., 2010). Similarly to Arabidopsis, rice plants silenced to both chloroplastic APX isoforms (APX7 and APX8) displayed no phenotype under both optimal and excessive light conditions (Caverzan et al., 2014). These data indicate possible compensatory mechanisms triggered in chloroplastic APX deficient plants, but routes involved remains to be clarified (Caverzan et al., 2014). These compensatory mechanisms, at least in Arabidopsis, probably involve up-expression of 2-cys peroxiredoxins (Kangasjärvi et al., 2008).

4. Remarks and further perspectives

During ~500 million years of evolution, land plants must develop several strategies to cope with oscillations in light intensity, sustain its photosynthetic activity and survive. Under the majority of environmental conditions, light provides more energy than the whole plant metabolism can use and, therefore, plants are commonly subjected to excessive light conditions. In the current review some important mechanisms of photoprotection are discussed, employing an integrative view, which encompass fast adjustments processes such as NPQ and ROS scavenging in chloroplasts and long-term acclimatory mechanisms, involving regulation of expression of several genes/proteins. An especial focus is given to chloroplastic APX, believed to be one of the most important peroxidases in chloroplasts.

As evidenced here, several reports concerning chloroplastic APX deficiency are not able to explain its importance. Complexity of systemic responses and gene/protein networks in leaf cells should have contributed in great part for this difficulty. Certainly, understanding the importance of other peroxidases such as peroxiredoxins (PRX) should be essential for further interpretation of such controversial results involving APX proteins. Moreover, in order to achieve a deeper knowledge regarding plant cell metabolism and, therefore, take more robust conclusion about the importance of each mechanism of photoprotection related to sustain photosynthesis under excessive light, new integrative approaches are needed.

Thus, plant photoprotective mechanisms can encompass foliar and chloroplastic movements, excess energy dissipation as heat, antioxidative responses, stomata modulation, Calvin cycle activity adjustment, photorespiration and even nitrogen assimilation metabolism. In other words, every reductant metabolic process in plant cells could, in principle, represents an important photoprotective mechanism under excessive light (Fig. 12). Therefore, use of integrated approaches, including a deeper systemic view, is crucial to further discoveries regarding plant light tolerance.

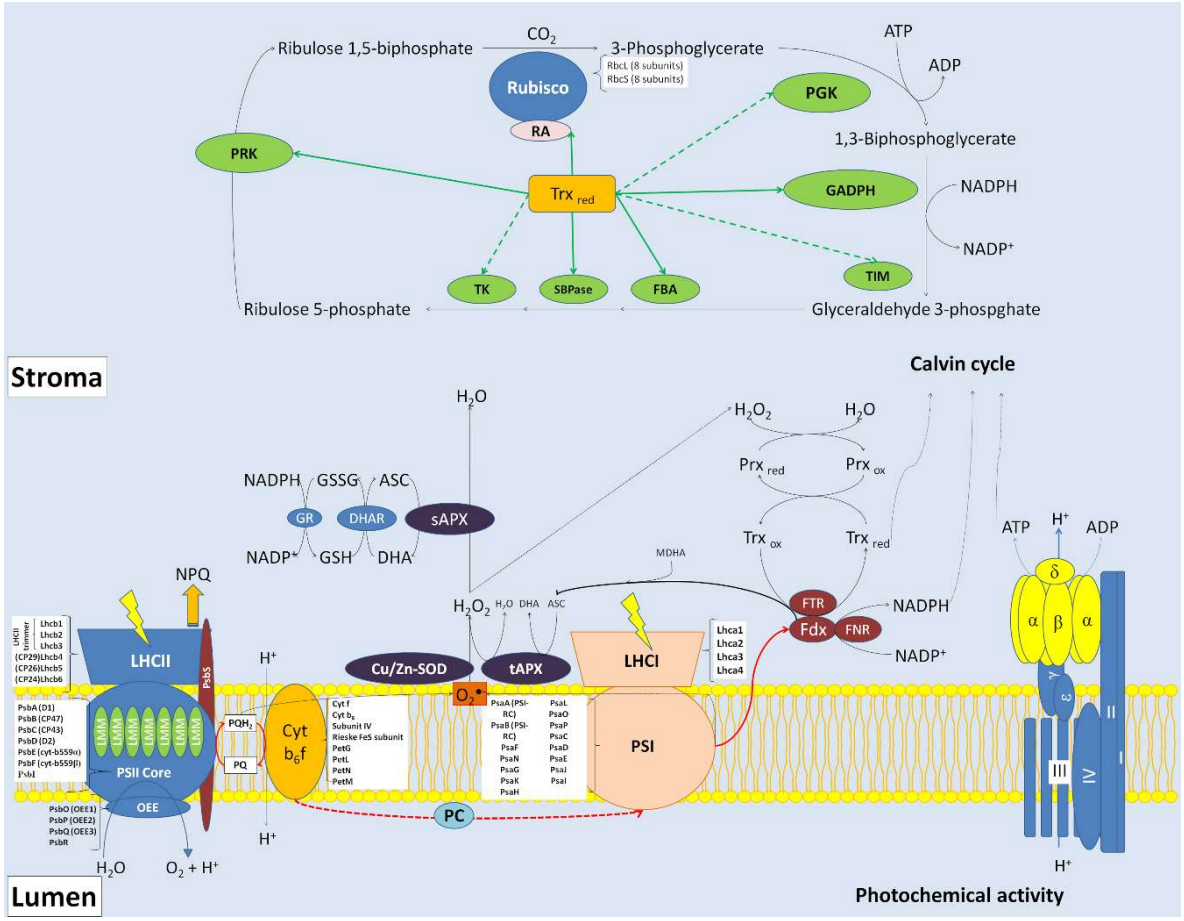

Figure 12. Simplified photosynthetic model containing the several components of photochemical (PSII and PSI) and Calvin cycle phases. The scheme shows the main photosynthesis proteins, including those related to redox reactions, which are commonly characterized by proteomics approaches. Abbreviations: ASC, ascorbate; Cu/Zn-SOD, Cu/Zn superoxide dismutase; Cyt b6f, cytochrome b6f; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; FBA, fructose biphosphate aldolase; FDX, ferredoxin; FNR, ferredoxin NADP(H) oxireductase; FTR, ferredoxin-thioredoxin reductase; GADPH, glyceraldehyde phosphate dehydrogenase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; LHCI, light harvest complex antenna of PSI; LHCII, light harvest complex antenna of PSII; LMM, PSII low molecular mass proteins; MDHA, monodehydroascorbate; NPQ, non-photochemical quenching; OEE, oxygen evolving enhancer proteins; PC, plastocyanin; PGK, phosphoglyceratekinase; PQ, oxidized plastoquinone; PQH_2, reduced plastoquinone; PRK, phosphoribulokinase; PRX_{ox}, oxidized peroxiredoxin; PRX_{red}, reduced peroxiredoxin; PSI, photosystem I; PSII, photosystem II; RA, rubisco activase; Rubisco, ribulose 1,5-biphosphate carboxylase/oxygenase; sAPX, stromal ascorbate peroxidase; SBPase, sedoheptulose biphosphate phosphatase; tAPX, thylakoidal ascorbate peroxidase; TIM, triose phosphate isomerase; TK, transketolase; TRX_{ox}, oxidized thioredoxin; TRX_{red}, reduced thioredoxin. Reproduced from Silveira and Carvalho (2016) - *Journal of Proteomics*.

References

Aarti PD, Tanaka R, Tanaka A (2006) Effects of oxidative stress on chlorophyll biosynthesis in cucumber (*Cucumis sativus*) cotyledons. Physiol Plant **128**: 186–197

Ahn TK, Avenson TJ, Ballottari M, Cheng Y-C, Niyogi KK, Bassi R, Fleming GR (2008) Architecture of a charge-transfer state regulating light harvesting in a plant antenna protein. Science **320**: 794–797

van Amerongen H, Croce R (2013) Light harvesting in photosystem II. Photosynth Res **116**: 251–263

Andersson J, Walters RG, Horton P, Jansson S (2001) Antisense inhibition of the photosynthetic antenna proteins CP29 and CP26: implications for the mechanism of protective energy dissipation. Plant Cell **13**: 1193–1204

Andersson J, Wentworth M, Walters RG, Howard CA, Ruban AV, Horton P, Jansson S (2003) Absence of the Lhcb1 and Lhcb2 proteins of the light-harvesting complex of photosystem II - Effects on photosynthesis, grana stacking and fitness. Plant J **35**: 350–361

Aro EM, McCaffery S, Anderson JM (1993) Photoinhibition and d1 protein degradation in peas acclimated to different growth irradiances. Plant Physiol **103**: 835–843

Asada K (1999) The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol **50**: 601–639

Baccar R, Fournier C, Dornbusch T, Andrieu B, Gouache D, Robert C (2011) Modelling the effect of wheat canopy architecture as affected by sowing density on Septoria tritici epidemics using a coupled epidemicvirtual plant model. Ann Bot **108**: 1179–1194

Badawi GH, Kawano N, Yamauchi Y, Shimada E, Sasaki R, Kubo A, Tanaka K (2004) Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. Physiol Plant **121**: 231–238

Bailey S, Walters RG, Jansson S, Horton P (2001) Acclimation of Arabidopsis thaliana to the light environment: the existence of separate low light and high light responses. Planta **213**: 794–801

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu Rev Plant Biol **59**: 89–113

Ballottari M, Dall'Osto L, Morosinotto T, Bassi R (2007) Contrasting behavior of higher plant photosystem I and II antenna systems during acclimation. J Biol Chem **282**: 8947–8958

Benson SL, Maheswaran P, Ware MA, Hunter CN, Horton P, Jansson S, Ruban AV, Johnson MP (2015) An intact light harvesting complex I antenna system is required for complete state transitions in Arabidopsis. Nat Plants **1**: 15176

de Bianchi S, Betterle N, Kouril R, Cazzaniga S, Boekema E, Bassi R, Dall'Osto L (2011) Arabidopsis mutants deleted in the light-harvesting protein Lhcb4 have a disrupted photosystem II macrostructure and are defective in photoprotection. Plant Cell **23**: 2659–2679

de Bianchi S, Dall'Osto L, Tognon G, Morosinotto T, Bassi R (2008) Minor antenna proteins CP24 and CP26 affect the interactions between photosystem II subunits and the electron transport rate in grana membranes of Arabidopsis. Plant Cell **20**: 1012–1028

Blankenship RE (2013) Molecular mechanisms of photosynthesis. John Wiley & Sons

Bonifacio A, Martins MO, Ribeiro CW, Fontenele AV, Carvalho FEL, Margis-Pinheiro M, Silveira JAG (2011) Role of peroxidases in the compensation of cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress. Plant, Cell Environ **34**: 1705–1722

Borisova-Mubarakshina MM, Ivanov BN, Vetoshkina DV, Lubimov VY, Fedorchuk TP, Naydov IA, Kozuleva M a, Rudenko NN, Dall'Osto L, Cazzaniga S, et al (2015) Long-term acclimatory response to excess excitation energy: evidence for a role of hydrogen peroxide in the regulation of photosystem II antenna size. J Exp Bot **66**: 7151–7164

Briantais JM, Vernotte C, Picaud M, Krause GH (1979) A quantitative study of the slow decline of chlorophyll a fluorescence in isolated chloroplasts. BBA - Bioenerg **548**: 128–138

Bricker TM, Roose JL, Fagerlund RD, Frankel LK, Eaton-Rye JJ (2012) The extrinsic proteins of Photosystem II. Biochim Biophys Acta - Bioenerg **1817**: 121–142

Burgess AJ, Retkute R, Pound MP, Foulkes J, Preston SP, Jensen OE, Pridmore TP, Murchie EH (2015) High-resolution three-dimensional structural data quantify the impact of photoinhibition on long-term carbon gain in wheat canopies in the field. Plant Physiol **169**: 1192–1204

Calvin M, Benson AA (1948) The path of carbon in photosynthesis. US Atomic Energy Commission, Technical Information Division

Carvalho FEL, Ware MA, Ruban AV (2015) Quantifying the dynamics of light tolerance in *Arabidopsis* plants during ontogenesis. Plant Cell Environ **38**: 2603–2617

Caverzan A, Bonifacio A, Carvalho FEL, Andrade CMB, Passaia G, Schünemann M, Maraschin F dos S, Martins MO, Teixeira FK, Rauber R, et al (2014) The knockdown

of chloroplastic ascorbate peroxidases reveals its regulatory role in the photosynthesis and protection under photo-oxidative stress in rice. Plant Sci **214**: 74–87

Chmeliov J, Bricker WP, Lo C, Jouin E, Valkūnas L, Ruban A, Duffy CDP (2015) An "all pigment" model of excitation quenching in LHCII. Phys Chem Chem Phys **17**: 15857–15867

Corpas FJ (2015) What is the role of hydrogen peroxide in plant peroxisomes? Plant Biol **17**: 1099–1103

Danna CH, Bartoli CG, Sacco F, Ingala LR, Santa-María GE, Guiamet JJ, Ugalde RA (2003) Thylakoid-bound ascorbate peroxidase mutant exhibits impaired electron transport and photosynthetic activity. Plant Physiol **132**: 2116–2125

Davletova S, Rizhsky L, Liang H, Shengqiang Z, Oliver DJ, Coutu J, Shulaev V, Schlauch K, Mittler R (2005) Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis. Plant Cell **17**: 268–281

Demmig-Adams B, Adams WW (1992) Photoprotection and other responses of plants to high light stress. Annu Rev Plant Physiol Plant Mol Biol **43**: 599–626

Demmig-Adams B, Cohu CM, Muller O, Adams WW (2012) Modulation of photosynthetic energy conversion efficiency in nature: From seconds to seasons. Photosynth Res **113**: 75–88

Desikan R, A-H-Mackerness S, Hancock JT, Neill SJ (2001) Regulation of the Arabidopsis transcriptome by oxidative stress. Plant Physiol **127**: 159–172

Farber A, Young AJ, Ruban AV, Horton P, Jahns P (1997) Dynamics of xanthophyll-cycle activity in different antenna subcomplexes in the photosynthetic membranes of higher plants (the relationship between zeaxanthin conversion and nonphotochemical fluorescence quenching). Plant Physiol **115**: 1609–1618

Ferreira-Silva SL, Voigt EL, Silva EN, Maia JM, Fontenele A V, Silveira JAG (2011) High temperature positively modulates oxidative protection in salt-stressed cashew plants. Environ Exp Bot **74**: 162–170

Fourcaud T, Zhang X, Stokes A, Lambers H, Kürner C (2008) Plant growth modelling and applications: The increasing importance of plant architecture in growth models. Ann Bot **101**: 1053–1063

Foyer CH, Neukermans J, Queval G, Noctor G, Harbinson J (2012) Photosynthetic control of electron transport and the regulation of gene expression. J Exp Bot **63**: 1637–1661

Foyer CH, Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant **119**: 355–364

Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol **155**: 2–18

Funk C, Schröder WP, Napiwotzki a, Tjus SE, Renger G, Andersson B (1995) The PSII-S protein of higher plants: A new type of pigment- binding protein. Biochemistry **34**: 11133–11141

Genty B, Briantais J-M, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta - Gen Subj **990**: 87–92

Giacomelli L, Masi A, Ripoll DR, Lee MJ, Van Wijk KJ (2007) *Arabidopsis thaliana* deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low. Plant Mol Biol **65**: 627–644

Giovagnetti V, Ruban AV (2015) Discerning the effects of photoinhibition and photoprotection on the rate of oxygen evolution in Arabidopsis leaves. J Photochem Photobiol B Biol **152**: 272-278

Goh CH, Ko SM, Koh S, Kim YJ, Bae HJ (2012) Photosynthesis and environments: photoinhibition and repair mechanisms in plants. J Plant Biol **55**: 93–101

Goss R, Lepetit B (2014) Biodiversity of NPQ. J Plant Physiol **172**:13-32

Govindjee, Munday JC, Papageorgiou GC (1966) Fluorescence studies with algae: Changes with time and pre-illumination. Brookhaven Symp Biol **19**: 434–445

Graßes T, Pesaresi P, Schiavon F, Varotto C, Salamini F, Jahns P, Leister D (2002) The role of ΔpH-dependent dissipation of excitation energy in protecting photosystem II against light-induced damage in *Arabidopsis thaliana*. Plant Physiol Biochem **40**: 41–49

Havaux M, Bonfils JP, Lütz C, Niyogi KK (2000) Photodamage of the photosynthetic apparatus and its dependence on the leaf developmental stage in the *npq1* Arabidopsis mutant deficient in the xanthophyll cycle. Plant Physiol **124**: 273–284

Hill R, Bendall F (1960) Function of the two cytochrome components in chloroplasts: a working hypothesis. Nature **186**: 136–137

Horton P, Ruban AV, Walters RG (1996) Regulation of light harvesting in green plants. Annu Rev Plant Physiol Plant Mol Biol **47**: 655–684

Horton P, Ruban A (2005) Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection. J Exp Bot **56**: 365–373

Jahns P, Holzwarth AR (2012) The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. Biochim Biophys Acta - Bioenerg **1817**: 182–193

Jahns P, Krause GH (1994) Xanthophyll cycle and energy-dependent fluorescence quenching

in leaves from pea plants grown under intermittent light. Planta **192**: 176–182

Johnson MP, Ruban AV (2011) Restoration of rapidly reversible photoprotective energy dissipation in the absence of PsbS protein by enhanced DeltapH. J Biol Chem **286**: 19973–19981

Kangasjärvi S, Lepistö A, Hännikäinen K, Piippo M, Luomala E-M, Aro E-M, Rintamäki E (2008) Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses. Biochem J **412**: 275–85

Kao W, Forseth I (1992) Dirunal leaf movement, chlorophyll fluorescence and carbon assimilation in soybean grown under different nitrogen and water availabilities. Plant, Cell Environ **15**: 703–710

Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux PM (1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. Plant Cell **9**: 627–640

Karpiński S, Szechyńska-Hebda M, Wituszyńska W, Burdiak P (2013) Light acclimation, retrograde signalling, cell death and immune defences in plants. Plant, Cell Environ **36**: 736–744

Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Miyao M, Wada M (2002) Chloroplast avoidance movement reduces photodamage in plants. Nature **420**: 829–832

Klein P, Seidel T, Stöcker B, Dietz K-J (2012) The membrane-tethered transcription factor ANAC089 serves as redox-dependent suppressor of stromal ascorbate peroxidase gene expression. Front Plant Sci **3**: 247

Kovács L, Damkjaer J, Kereïche S, Ilioaia C, Ruban AV, Boekema EJ, Jansson S, Horton P (2006) Lack of the light-harvesting complex CP24 affects the structure and function of the grana membranes of higher plant chloroplasts. Plant Cell **18**: 3106–3120

Kramer D, Johnson G, Kiirats O, Edwards G (2004) New fluorescence parameters for the determination of Q_A redox state and excitation energy fluxes. Photosynth Res **79**: 209–218

Krause GH (1974) Changes in chlorophyll fluorescence in relation to light-dependent cation transfer across thylakoid membranes. BBA - Bioenerg **333**: 301–313

Kriedeman PE, Neales TF, Ashton DH (1964) Photosynthesis in relation to leaf orientation and light interception. Aust J Biol Sci **17**: 591–600

Li X-P, Muller-Moule P, Gilmore AM, Niyogi KK (2002) PsbS-dependent enhancement of feedback de-excitation protects photosystem II from photoinhibition. Proc Natl Acad Sci USA **99**: 15222–15227

Li Z, Wakao S, Fischer BB, Niyogi KK (2009a) Sensing and responding to excess light. Annu

Rev Plant Biol **60**: 239–260

Li Y-JJ, Hai R-LL, Du X-HH, Jiang X-NN, Lu H (2009b) Over-expression of a Populus peroxisomal ascorbate peroxidase (PpAPX) gene in tobacco plants enhances stress tolerance. Plant Breed **128**: 404–410

Maruta T, Tanouchi A, Tamoi M, Yabuta Y, Yoshimura K, Ishikawa T, Shigeoka S (2010) Arabidopsis chloroplastic ascorbate peroxidase isoenzymes play a dual role in photoprotection and gene regulation under photooxidative stress. Plant Cell Physiol **51**: 190–200

Matsubara S, Chow WS (2004) Populations of photoinactivated photosystem II reaction centers characterized by chlorophyll a fluorescence lifetime *in vivo*. Proc Natl Acad Sci USA **101**: 18234–18239

Mehler AH (1951) Studies on reactions of illuminated chloroplasts. II. Stimulation and inhibition of the reaction with molecular oxygen. Arch Biochem Biophys **34**: 339–351

Mhamdi A, Noctor G, Baker A (2012) Plant catalases: peroxisomal redox guardians. Arch Biochem Biophys **525**: 181–194

Mhamdi A, Queval G, Chaouch S, Vanderauwera S, van Breusegem F, Noctor G (2010) Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models. J Exp Bot **61**: 4197–4220

Mubarakshina MM, Ivanov BN (2010) The production and scavenging of reactive oxygen species in the plastoquinone pool of chloroplast thylakoid membranes. Physiol Plant **140**: 103–110

Mubarakshina MM, Ivanov BN, Naydov IA, Hillier W, Badger MR, Krieger-Liszkay A (2010) Production and diffusion of chloroplastic H_2O_2 and its implication to signalling. J Exp Bot **61**: 3577–3587

Munné-bosch S, Queval G, Foyer CH (2013) the impact of global change factors on redox signaling underpinning stress tolerance 1. Plant Physiol **161**: 5–19

Murata N, Sugahara K (1969) Control of excitation transfer in photosynthesis III. Light-induced decrease of chlorophyll a fluorescence related to photophosphorylation system in spinach chloroplasts. Biochem Biophys Acta **189**: 182–192

Murchie EH, Ali A, Herman T (2015) Photoprotection as a trait for rice yield improvement: status and prospects. Rice **8**: 31

Murchie EH, Chen Y, Hubbart S, Peng S, Horton P (1999) Interactions between senescence and leaf orientation determine in situ patterns of photosynthesis and photoinhibition in field-grown rice. Plant Physiol **119**: 553–564

Murchie EH, Hubbart S, Peng S, Horton P (2005) Acclimation of photosynthesis to high irradiance in rice: Gene expression and interactions with leaf development. J Exp Bot **56**: 449–460

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbato specific peroxidase in spinach chloroplasts. Plant Cell Physiol **22**: 867–880

Nilkens M, Kress E, Lambrev P, Miloslavina Y, Müller M, Holzwarth AR, Jahns P (2010) Identification of a slowly inducible zeaxanthin-dependent component of non-photochemical quenching of chlorophyll fluorescence generated under steady-state conditions in Arabidopsis. Biochim Biophys Acta - Bioenerg **1797**: 466–475

Nishiyama Y, Allakhverdiev SI, Murata N (2011) Protein synthesis is the primary target of reactive oxygen species in the photoinhibition of photosystem II. Physiol Plant **142**: 35–46

Niyogi KK, Li X-P, Rosenberg V, Jung H-S (2005) Is PsbS the site of non-photochemical quenching in photosynthesis? J Exp Bot **56**: 375–382

Niyogi KK, Shih C, Soon Chow W, Pogson BJ, Dellapenna D, Björkman O (2001) Photoprotection in a zeaxanthin- and lutein-deficient double mutant of Arabidopsis. Photosynth Res **67**: 139–145

Oikawa K, Kasahara M, Kiyosue T, Kagawa T, Suetsugu N, Takahashi F, Kanegae T, Niwa Y, Kadota A, Wada M (2003) Chloroplast unusual positioning1 is essential for proper chloroplast positioning. Plant Cell **15**: 2805–2815

Pagliano C, Saracco G, Barber J (2013) Structural, functional and auxiliary proteins of photosystem II. Photosynth Res **116**: 167–188

Pastenes C, Pimentel P, Lillo J (2005) Leaf movements and photoinhibition in relation to water stress in field-grown beans. J Exp Bot **56**: 425–433

Peter GF, Thornber JP (1991) Biochemical composition and organization of higher plant photosystem II light-harvesting pigment-proteins. J Biol Chem **266**: 16745–16754

Pospísil P (2009) Production of reactive oxygen species by photosystem II. Biochim Biophys Acta **1787**: 1151–1160

Pospíšil P (2014) The role of metals in production and scavenging of reactive oxygen species in photosystem II. Plant Cell Physiol **55**: 1224–1232

Puthiyaveetil S, Ibrahim IM, Allen JF (2012) Oxidation-reduction signalling components in regulatory pathways of state transitions and photosystem stoichiometry adjustment in chloroplasts. Plant, Cell Environ **35**: 347–359

Raven JA (1984) Physiological correlates of the morphology of early vascular plants. Bot J

Linn Soc **88**: 105–126

Ruban AV (2015) Evolution under the sun: Optimizing light harvesting in photosynthesis. J Exp Bot **66**: 7–23

Ruban AV (2016) Non-photochemical chlorophyll fluorescence quenching: mechanism and effectiveness in protection against photodamage. Plant Physiol **70**: 1903–1916

Ruban AV, Murchie EH (2012) Assessing the photoprotective effectiveness of non-photochemical chlorophyll fluorescence quenching: A new approach. Biochim Biophys Acta - Bioenerg **1817**: 977–982

Ruban AV, Belgio E (2014) The relationship between maximum tolerated light intensity and photoprotective energy dissipation in the photosynthetic antenna: chloroplast gains and losses. Philos Trans R Soc B Biol Sci **369**: 20130222–20130222

Ruban AV, Rees D, Pascal AA, Horton P (1992) Mechanism of ΔpH-dependent dissipation of absorbed excitation energy by photosynthetic membranes. II. The relationship between LHCII aggregation *in vitro* and qE in isolated thylakoids. Biochim Biophys Acta - Bioenerg **1102**: 39–44

Ruban AV, Young AJ, Horton P (1993) Induction of nonphotochemical energy dissipation and absorbance changes in leaves (evidence for changes in the state of the light-harvesting system of photosystem II *in vivo*). Plant Physiol **102**: 741–750

Ruban AV, Lee PJ, Wentworth M, Young AJ, Horton P (1999) Determination of the stoichiometry and strength of binding of xanthophylls to the photosystem II light harvesting complexes. J Biol Chem **274**: 10458–10465

Ruban AV, Berera R, Ilioaia C, van Stokkum IHM, Kennis JTM, Pascal AA, van Amerongen H, Robert B, Horton P, van Grondelle R (2007) Identification of a mechanism of photoprotective energy dissipation in higher plants. Nature **450**: 575–578

Ruban AV, Johnson MP, Duffy CDP (2012) The photoprotective molecular switch in the photosystem II antenna. Biochim Biophys Acta - Bioenerg **1817**: 167–181

Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, Briggs WR, Wada M, Okada K (2001) Arabidopsis nph1 and npl1: blue light receptors that mediate both phototropism and chloroplast relocation. Proc Natl Acad Sci USA **98**: 6969–6974

Shen J-R, Henmi T, Kamiya N (2008) Structure and function of photosystem II. In: Fromme P (eds) Photosynthetic Protein Complexes. Wiley-VCH Verlag GmbH & Co. KGaA. pp. 83–106

Shigeoka S, Maruta T (2014) Cellular redox regulation, signaling, and stress response in plants. Biosci Biotechnol Biochem **78**: 1457–1470

Silva EN, Silveira JA G, Ribeiro RV, Vieira SA (2015) Photoprotective function of energy dissipation by thermal processes and photorespiratory mechanisms in *Jatropha curcas* plants during different intensities of drought and after recovery. Environ Exp Bot **110**: 36–45

Silveira JAG, Carvalho FEL (2016) Proteomics, photosynthesis and salt resistance in crops: An integrative view. J Proteomics. doi: 10.1016/j.jprot.2016.03.013

Singh N, Mishra A, Jha B (2014) Over-expression of the peroxisomal ascorbate peroxidase (SbpAPX) gene cloned from halophyte *Salicornia brachiata* confers salt and drought stress tolerance in transgenic tobacco. Mar Biotechnol (NY) **16**: 321–332

Ślesak I, Libik M, Karpinska B, Karpinski S, Miszalski Z (2007) The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. Acta Biochim Pol **54**: 39–50

Smirnoff N (2000) Ascorbate biosynthesis and function in photoprotection. Philos Trans R Soc Lond B Biol Sci **355**: 1455–1464

Souza GM, Lüttge U (2015) Stability as a phenomenon emergent from plasticity–complexity–diversity. in: Eco-physiology. pp 211–239

Strand Å (2004) Plastid-to-nucleus signalling. Curr Opin Plant Biol **7**: 621–625

Strand A, Asami T, Alonso J, Ecker JR, Chory J (2003) Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. Nature **5**: 79–83

Suzuki N, Miller G, Salazar C, Mondal HA, Shulaev E, Cortes DF, Shuman JL, Luo X, Shah J, Schlauch K, et al (2013) Temporal-spatial interaction between reactive oxygen species and abscisic acid regulates rapid systemic acclimation in plants. Plant Cell **25**: 3553–3569

Takahashi S, Badger MR (2011) Photoprotection in plants: A new light on photosystem II damage. Trends Plant Sci **16**: 53–60

Teixeira FK, Menezes-Benavente L, Galvão VC, Margis R, Margis-Pinheiro M (2006) Rice ascorbate peroxidase gene family encodes functionally diverse isoforms localized in different subcellular compartments. Planta **224**: 300–314

Teixeira FK, Menezes-Benavente L, Margis R, Margis-Pinheiro M (2004) Analysis of the molecular evolutionary history of the ascorbate peroxidase gene family: Inferences from the rice genome. J Mol Evol **59**: 761–770

Timperio AM, Gevi F, Ceci LR, Zolla L (2012) Acclimation to intense light implies changes at the level of trimeric subunits involved in the structural organization of the main light-harvesting complex of photosystem II (LHCII) and their isoforms. Plant Physiol Biochem

50: 8–14

Umena Y, Kawakami K, Shen J-R, Kamiya N (2011) Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. Nature **473**: 55–60

Voss I, Sunil B, Scheibe R, Raghavendra AS (2013) Emerging concept for the role of photorespiration as an important part of abiotic stress response. Plant Biol (Stuttg) **15**: 713–722

Ware MA, Belgio E, Ruban AV (2014) Comparison of the protective effectiveness of NPQ in Arabidopsis plants deficient in PsbS protein and zeaxanthin. J Exp Bot **66**: 1259–1270

Wellman CH, Osterloff PL, Mohiuddin U (2003) Fragments of the earliest land plants. Nature **425**: 282–285

Wraight CA, Crofts, AR (1970) Energy-dependent quenching of chlorophyll *a* fluorescence in isolated chloroplasts. Eur J Biochem **17**: 319–327

Zivcak M, Brestic M, Kalaji HM, Govindjee (2014) Photosynthetic responses of sun- and shade-grown barley leaves to high light: Is the lower PSII connectivity in shade leaves associated with protection against excess of light? Photosynth Res **119**: 339–354

Zulfugarov IS, Tovuu A, Eu Y-J, Dogsom B, Poudyal RS, Nath K, Hall M, Banerjee M, Yoon UC, Moon Y-H, et al (2014) Production of superoxide from Photosystem II in a rice (*Oryza sativa* L.) mutant lacking PsbS. BMC Plant Biol **14**: 242

CHAPTER II

Fabricio E.L. Carvalho

Plant, Cell & Environment

Plant, Cell and Environment (2015) **38**, 2603–2617 doi: 10.1111/pce.12574

Original Article

Quantifying the dynamics of light tolerance in *Arabidopsis* plants during ontogenesis

Fabricio E. L. Carvalho, Maxwell A. Ware & Alexander V. Ruban

School of Biological and Chemical Sciences, Queen Mary University of London, London E1 4NS, UK

ABSTRACT

The amount of light plants can tolerate during different phases of ontogenesis remains largely unknown. This was addressed here employing a novel methodology that uses the coefficient of photochemical quenching (qP) to assess the intactness of photosystem II reaction centres. Fluorescence quenching coefficients, total chlorophyll content and concentration of anthocyanins were determined weekly during the juvenile, adult, reproductive and senescent phases of plant ontogenesis. This enabled quantification of the protective effectiveness of non-photochemical fluorescence quenching (NPQ) and determination of light tolerance. The light intensity that caused photoinhibition in 50% of leaf population increased from ~70 μmol m^{-2} s^{-1}, for 1-week-old seedlings, to a maximum of 1385 μmol m^{-2} s^{-1} for 8-week-old plants. After 8 weeks, the tolerated light intensity started to gradually decline, becoming only 332 μmol m^{-2} s^{-1} for 13-week-old plants. The dependency of light tolerance on plant age was well-related to the amplitude of protective NPQ (pNPQ) and the electron transport rates (ETRs). Light tolerance did not, however, show a similar trend to chlorophyll *a*/*b* ratios and content of anthocyanins. Our data suggest that pNPQ is crucial in defining the capability of high light tolerance by *Arabidopsis* plants during ontogenesis.

Key-words: photoinhibition; protective NPQ.

INTRODUCTION

Plants are sessile organisms, and due to this nature they have to constantly adapt to a variety of dynamically changing environmental factors (Raven 1984). When growing in the shade, they need to absorb the greatest amount of light energy available to maximize photosynthesis (Ruban 2015). To cope with this problem, evolutionary pressures resulted in the emergence of highly specialized pigment–protein complexes that form the light-harvesting antenna, which efficiently supplies the photosynthetic reaction centres reaction center II (RCII) with photons (Peter & Thornber 1991; Boekema *et al.* 1995; van Amerongen & Croce 2013). The antenna of higher plants photosystem II (PSII) is composed of several trimeric major antenna complexes light harvesting complex II (LHCII) and three minor complexes (CP24, CP26 and CP29) that bind chlorophylls *a* and *b* and xanthophylls (Bassi *et al.* 1987; Peter & Thornber 1991; Bassi & Dainese 1992; Ruban *et al.* 1999; Hogewoning *et al.* 2012). RCII contains specialized chlorophyll molecules, collectively named P680, where charge separation takes place in coordination with the splitting of water in the oxygen evolving complex (OEC) (Barber 2002; Umena *et al.* 2011). Electrons from RCII are transported by a chain of electron/proton carriers in the thylakoid membrane. This generates a redox- and proton energy gradient, which is employed in the production of NADPH and ATP, with these subsequently used in the Calvin cycle for CO_2 assimilation (Arnon *et al.* 1954; Hill & Bendall 1960; Paul & Foyer 2001).

The electron transport and formation of ATP and NADPH proceed at much slower rates than the light harvesting and energy transfer to the RCII by antennae (Ruban & Murchie 2012; Ruban *et al.* 2012). As an immediate consequence, the amount of energy absorbed recurrently overcomes the metabolic energy demands. This leads to accumulation of excess energy in the thylakoid membrane that can potentially be harmful to RCII (Aro *et al.* 1993; Adams *et al.* 2006). The latter can lead to the permanent closure of reaction center (RCs), commonly known as photoinhibition, and the eventual formation of reactive oxygen species (ROS) that cause further damage to the photosynthetic membrane (Foyer & Shigeoka 2011; Nishiyama *et al.* 2011). To protect the membrane against photoinhibition, several processes have evolved in plants. Amongst them, the most prominent is related to a biochemical and biophysical mechanism capable of dissipating the energy excess in PSII as heat, called non-photochemical chlorophyll fluorescence quenching (NPQ) (Wraight & Crofts 1970; Briantais *et al.* 1979).

NPQ is composed of four major processes: state transition (qT), photoinhibition (qI), heat dissipation (qE) and zeaxanthin-dependent (qZ) quenching (Baker 2008; Jahns & Holzwarth 2012; Ruban & Murchie 2012; Ruban *et al.* 2012). qT is associated with redistributing energy absorption between the two photosystems, which is achieved by the partial detachment and migration of LHCII between PSII and photosystem I (PSI) (Murata 1969; Bellafiore *et al.* 2005). qI is a slowly reversible process and is the consequence of photoinhibitory events reflecting the damage to RCII, where closed RCII confers protection for open ones (Matsubara & Chow 2004). The major and fastest component of NPQ, qE, is believed to act in RCII photoprotection and is related to the dissipation of excess energy as heat (Baker 2008; Ruban &

Correspondence: A. V. Ruban. Fax: +44(0)2078827732; e-mail: a.ruban@qmul.ac.uk

Murchie 2012; Ruban *et al*. 2012). More recently described and with a formation/relaxation lifetime in the order of 10–15 min, qZ is believed to be correlated with the synthesis and epoxidation of zeaxanthin and is also related to photoprotection (Nilkens *et al*. 2010; Jahns & Holzwarth 2012).

The site of qE formation is believed to be the light-harvesting antenna of PSII, triggered by ΔpH and enhanced by violaxanthin de-epoxidation to zeaxanthin (Demmig-Adams & Adams 1992; Horton & Ruban 1992). The protein PsbS was discovered to play a crucial role in sensing lumen acidification and transducing the signal to the antenna (Li *et al*. 2000, 2002; Niyogi *et al*. 2005); however, the mechanism underlying the action of PsbS is not completely understood. Currently, the most consistent ideas concerning qE are that (1) there are three possible models for the site of qE: energy transfer by interactions between chlorophyll molecules (Horton *et al*. 1996), quenching interactions between the lutein bound at the L1 site and chlorophylls a612–a611–a610 (Ruban *et al*. 2007), and xanthophyll–chlorophyll quenching interactions between zeaxanthin bound at the L2 site and chlorophylls A5 and B5 (Ahn *et al*. 2008); (2) the increase in the lumen pH is the key event for triggering qE (Krause 1974; Horton & Ruban 1992); (3) the role of zeaxanthin (Demmig-Adams & Adams 1992) and PsbS (Funk *et al*. 1995; Li *et al*. 2002; Niyogi *et al*. 2005) is, by binding to antenna proteins, to increase the sensitivity of the membrane to pH changes, producing a new state of aggregation of light-harvesting complexes that allow the excess energy dissipation as heat (Horton & Ruban 1992; Färber *et al*. 1997; Ruban *et al*. 1999); and (4) structural changes play a vital role with aggregated LHCII promoting more efficient excess energy dissipation as heat (Horton & Ruban 1992; Betterle *et al*. 2009; Johnson & Ruban 2011). The use of PAM fluorimetry has been a useful tool to help monitor NPQ in plants and algae in response to various environmental and endogenous conditions (Hideg *et al*. 2008; Ivanov *et al*. 2012; Ruban 2015).

During plant ontogenesis, several physiological changes take place in the shoots, especially regarding the leaf area (Hopkinson 1964), dry weight (Milthorpe 1959) and thickness (Avery 1933). In *Arabidopsis thaliana*, the emergence of the radicle, hypocotyl and cotyledon occurs around 4–5 d after the germination and is followed by a succession of distinct phases regarding shoot growth (Boyes *et al*. 2001). The juvenile phase consists of vegetative growth, where the leaves are smaller and rounded, with trichomes on the adaxial side and a lack of serrations along the margin (Kerstetter & Poethig 1998; Tsukaya 2013). The adult phase is characterized by rosette growth, in terms of number of leaves and total diameter (Boyes *et al*. 2001). The leaves are larger and elongated; the trichomes are present on both adaxial and abaxial surfaces; and the serrations along the margin are frequent (Kerstetter & Poethig 1998; Tsukaya 2013). The reproductive phase is marked by inflorescence and is strongly regulated by the photoperiod prehistory (Amasino 2010). The last phase (senescence) marks the end of the plant life cycle and is recognized by the gradual appearance of necrosis on the leaves, followed by abscission and plant death (Noodén 1988; Matile *et al*. 1992; Woo *et al*. 2001).

Despite this relatively well-established developmental pattern, there are several environmental aspects capable of affecting normal ontogenesis, especially light availability (Woo *et al*. 2001; Schurr *et al*. 2006). Plants growing under high light present smaller rosette diameters, thicker leaves (Tsukaya 2013) and an earlier reproductive phase (Bailey *et al*. 2001). The long-term high light response is also associated with several physiological and molecular alterations related to photosynthesis, such as an increased chlorophyll *a*/*b* ratio, reduced light-harvesting antenna size and increase in anthocyanin content (Bailey *et al*. 2001; Ballottari *et al*. 2007; Kouřil *et al*. 2013; Wientjes *et al*. 2013). In the short term, high light stress on seedlings was also addressed in a few studies (Krause *et al*. 1995; Dodd *et al*. 1998; Manetas *et al*. 2002). These revealed that some plant species exhibit higher photoinhibition at juvenile phases (Krause *et al*. 1995), whilst other species present higher photoinhibition at the mature phase (Manetas *et al*. 2002). The high light stress effects on *Arabidopsis* seedlings, however, have been less studied, especially regarding the effects on photosynthesis. Moreover, previous studies have shown that the response to other abiotic stresses is dependent on the developmental stage of the plant, as observed by the extreme sensitivity of *Arabidopsis* seedlings to salt and oxidative/methyl viologen stress (Borsani *et al*. 2001).

Growth and photosynthesis are two of the most important processes that support plants' acclimation to stressful environments, especially light oscillations (Schurr *et al*. 2006). Indeed, both are regulated by endogenous and environmental processes and are considered to be very closely related (Schurr *et al*. 2006). The ratio and total content of chlorophyll *a* and *b*, and the number of stomata are known to vary remarkable during ontogenesis (Oquist *et al*. 1982; Krause *et al*. 1995; Dodd *et al*. 1998). Three main techniques have been employed to verify ontogenetic effects on photosynthesis: (1) the determination of F_v/F_m (Siffel *et al*. 1993; Barker *et al*. 1997; la Porta *et al*. 2006; Radochová & Tichá 2008; Liu *et al*. 2009); (2) content of protein D1 (Thiele *et al*. 1997; Nath *et al*. 2013) and (3) CO_2 assimilation (Oquist *et al*. 1982; la Porta *et al*. 2006; Radochová & Tichá 2008). There are, however, certain limitations to the understanding of the dynamics of photosynthesis during plant ontogenesis with these techniques. The F_v/F_m parameter, although a fast, non-invasive and consistent tool to estimate the integrity of the RCII, requires long periods of dark adaption, which is a barrier when estimating photosynthesis dynamics under fluctuating light environments (Ruban & Murchie 2012; Ware *et al*. 2015). The biochemical quantification of the D1 protein allows for the *in vitro* assessment of the integrity of RCII; however, it is a destructive technique requiring the abolition of light treatment (Ruban & Belgio 2014). The measurement of CO_2 assimilation, in turn, occurs too far from the site of photoinhibition and much slower than light harvesting and energy transfer to the RCII by antennae (Ruban 2015). Thus, the use of new approaches to assess the onset of photoinhibition in fluctuating light environments during leaf ontogenesis is essential.

In this study, a new methodology, consisting of the quantum coefficient of photochemical quenching (qP) measurements in the dark (qPd), was used. This allowed the quantification of the levels of light capable of inducing photoinhibition in leaves of *A. thaliana* from seedlings through to the final senescent phases of growth (Ruban & Murchie 2012; Ruban & Belgio 2014; Ware *et al.* 2015). Our results indicated that light tolerance is strongly dependent on plant age and the leaf developmental stage. Moreover, the light tolerance variation during ontogenesis was well related to protective NPQ (pNPQ) efficiency, electron transport rate (ETR) and total chlorophyll content, but not related to chlorophyll *a*/*b* ratios or anthocyanin concentration. These results offer new insights into the roles of NPQ, working together with metabolic demands, as the determinant factors in light tolerance during the different phases of plant ontogenesis.

MATERIALS AND METHODS

Plant growth

A. thaliana plants, ecotype Col-0, were sown onto 0.3 L pots containing a 6:6:1 mixture of soil, potting compost (John Innes Manufacturers Association, Berkshire, UK) and perlite. The germination occurred in a growth chamber with 24 °C (day)/18 °C (night), 45% humidity, 100 ± 20 μmol m^{-2} s^{-1} actinic light and photoperiod of 10 h. After 1 week the plants were transferred to growth shelves with 24 °C (day)/18 °C (night), 45% humidity, 175 ± 20 μmol m^{-2} s^{-1} actinic light and the same photoperiod. Plants were grown under these conditions for up to 13 weeks. For the collection of chlorophyll *a* fluorescence measurements, a total of 30 leaves from independent plants were used. The leaves were selected weekly, and in every measurement, only the leaves positioned externally on the rosette were utilized (Fig. 1d). The same *criteria* were adopted for collecting the leaves used for pigment determinations. Additionally, a second experiment was performed with 30 leaves from independent plants at different physiological phases in the same rosette (according to their position more external, intermediate or more internal in relation to the centre of the rosette).

Principle of pNPQ methodology

Decrease in the PSII yield upon illumination is related to two distinct processes: the increase of the reversible photoprotective component of NPQ (pNPQ) and the non-reversible photoinhibition of RCII. The equation derived by Ruban & Murchie (2012) allows the correlation of PSII yield (ΦPSII) with NPQ and photoinhibition in the dark following a period of light exposure:

$$\Phi PSII = \frac{qPd \cdot Fv/Fm}{\left[1 + \left(1 - \frac{Fv}{Fm}\right) \cdot NPQ\right]}. \quad (1)$$

The qPd values were obtained from the following equation:

$$qPd = \frac{Fm' - Fo'act.}{Fm' - Fo'calc.} \quad (2)$$

where Fm' is the maximum fluorescence yield after actinic light exposure; $Fo'_{act.}$ is the minimum fluorescence yield

Figure 1. Visual aspect of *Arabidopsis thaliana* at juvenile, adult, reproductive and senescent phases. The juvenile phase was represented by plants aged (a) 1 and (b) 2 weeks old. The adult phase was represented by plants aged (c) 4 and (d) 6 weeks old. The reproductive phase was represented by plants aged (e) 7 and (f) 8 weeks old. The senescent phase was represented by plants aged (g) 11 and (h) 13 weeks old.

measured in the dark following an illumination period, and $Fo'_{calc.}$ is the minimum fluorescence yield in the dark calculated according to Oxborough & Baker (1997):

$$Fo'calc. = \frac{1}{\frac{1}{Fo} - \frac{1}{Fm} + \frac{1}{Fm'}} \quad (3)$$

where Fo and Fm are minimum and the maximum fluorescence levels in dark-adapted leaves. In the absence of photoinhibition, there are no differences between the $Fo'_{act.}$ and $Fo'_{calc.}$, and as a consequence qPd is equal to 1. However, at high light, the RCII population becomes increasingly closed. This causes a deviation between $Fo'_{act.}$ and $Fo'_{calc.}$, as damaged RCII contributes to the minimum level of fluorescence ($Fo'_{act.}$). Reductions in qPd values below 0.98 are consistent with the deviations of actual yield of PSII from the theoretical PSII yield. These criteria provide a quick and efficient way of monitoring the early signs of photoinhibition *in vivo*. pNPQ = NPQ, when there is no photoinhibition at a given actinic light intensity (Ruban & Belgio 2014; Ware *et al.* 2015).

Chlorophyll *a* fluorescence measurements

The chlorophyll *a* fluorescence measurements were performed as described previously (Ruban & Belgio 2014; Ware *et al.* 2015). Plants aged between 1 and 13 weeks were dark-adapted for 45 min with the most external leaves being subjected to the procedure. The measures were obtained with a JUNIOR-PAM fluorimeter (Walz, Effeltrich, Germany), using a procedure of ~42 min. Thirty different plants were subjected to three different sets of qPd determination procedures of one of the following actinic light regimes: 0, 90, 190, 285, 420, 625, 820, 1150, 1500 μmol m^{-2} s^{-1}; 0, 81, 171, 256.5, 378, 562.5, 738, 1035, 1350 μmol m^{-2} s^{-1}; or 0, 72, 152, 228, 336, 500, 656, 920, 1200 μmol m^{-2} s^{-1}. Each procedure was performed 10 times for qPd measurements. The procedures start with the following scheme: (ML on/FR on)–(30 s)–(SP)–(30 s)–(SP)–(5 s), where ML = measuring light, FR = far red light; SP = saturating pulse (6000 μmol m^{-2} s^{-1} for 0.6 s). PSII maximum quantum efficiency (F_v/F_m, where $F_v = F_m - F_o$) was determined with the first SP. Subsequently, for light measurements, the following scheme was employed: (AL on/FR off)–(120 s)–(SP)–(180 s)–(SP)–(AL off), where AL = actinic light. The NPQ [NPQ = $(F_m/F_m') - 1$] and ETR [ETR = $F_v/F_m' \times \text{PPFD} \times 0.84 \times 0.5$] values were calculated from the second SP. qPd was measured suddenly after actinic light was turned off. For qPd measurements the following scheme was employed: (FR on)–(10 s)–(SP)–(5 s)–(AL on/FR off). The procedures were repeated with increasing AL intensities (Ruban & Murchie 2012; Ruban & Belgio 2014; Ware *et al.* 2015).

Total chlorophyll determination

The determination of total chlorophyll content and chlorophyll *a*/*b* ratios was performed according to Porra *et al.* (1989). Fresh leaf material (50–150 mg) from plants aged between 1 and 13 weeks was first homogenized in 80% ice cold acetone. The homogenate was centrifuged at 14 000 *g* for 5 min. The supernatant obtained was used for spectrophotometric quantification of chlorophyll *a* and *b*. The absorbance was measured in two wavelengths, 663 and 646 nm (Porra *et al.* 1989). The chlorophyll content was expressed as microgram chlorophyll per square centimetre.

Determination of the content of anthocyanins

The total anthocyanins were determined as previously described (Neff & Chory 1998). Fresh leaf material (50–150 mg) was first reduced to a fine powder in the presence of liquid nitrogen. Three hundred microlitres of methanol and 1% HCl were added with the homogenate remaining at 4 °C overnight. Subsequently, 200 μL of H_2O and 500 μL of chloroform were added to the homogenate and centrifuged at 14 000 *g* for 5 min. After centrifugation, two different fractions were obtained. The bottom fraction consisted mainly of chlorophylls and other pigments, and the upper fraction anthocyanins (Neff & Chory 1998). The upper fraction was used for spectrophotometric quantification of total anthocyanins. The absorbance was measured in two wavelengths, 530 and 657 nm. The total content of anthocyanins was expressed as A530–A657 g FW^{-1}.

ROS quantification assays

The lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) in accordance with Cakmak & Horst (1991). The concentration of TBARS was calculated using its absorption coefficient (155 mM^{-1} cm^{-1}), and the results are expressed as ηmol MDA-TBA g FW^{-1}. *In situ* detection of hydrogen peroxide (H_2O_2) was performed by staining with 3,3′-diaminobenzidine (DAB) as previously described (Thordal-Christensen *et al.* 1997). Leaf segments were infiltrated under dark conditions with a 0.1% (w/v) DAB solution. The segments were incubated for approximately 16 h in dark conditions and then destained with 0.15% (w/v) trichloroacetic acid in an ethanol/chloroform solution (4:1 v/v) for 48 h before being photographed.

Statistical analysis

The comparisons between the averages of pNPQ formation, chlorophyll content, chlorophyll *a*/*b* ratio, anthocyanin content, F_v/F_m and ETR values were statistically analysed by an analysis of variance (ANOVA) test, followed by Tukey's test, with a 5% confidence interval ($P \leq 0.05$). The tests were performed using SigmaPlot12 (Systat Software, Inc., Chicago, IL, USA).

The *Z*-test was used to statistically compare the differences in range and amplitude of light intensity which caused photoinhibition in 50% of leaf populations:

$$Z = \frac{(Average\ 1 - Average\ 2)}{SD\ difference} \quad (4)$$

where average 1 and average 2 corresponds to the different light intensities which caused photoinhibition in 50% of leaf populations at any two given plant ages. This was calculated from the sigmoidal, Hill, three parameter ($f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software). The standard deviation (SD) difference was calculated according with the following equation:

$$\text{SD difference} = \sqrt{(\text{SD1})^2 + (\text{SD2})^2} \quad (5)$$

where SD1 and SD2 represent the standard deviation of two different light intensities which caused photoinhibition in 50% of leaf populations at any two given plant ages. This was also calculated using SigmaPlot12 (Systat Software). The Z values equal to or above 1.96 (which represents $P = 0.05$) were considered statistically significant.

For all the chlorophyll *a* fluorescence determinations, 30 independent leaves, selected amongst the most externally positioned in rosette, were used ($n = 30$). For the determination of chlorophyll content, chlorophyll *a*/*b* ratio and anthocyanin content, six independent leaves (also following the same criteria described above) were utilized ($n = 6$). For *in situ* H_2O_2 detection and TBARS, three leaves amongst the most external in the rosette from independent plants were used.

RESULTS

NPQ formation and qPd dynamics are age dependent

Four different phases of development were evaluated in *Arabidopsis* plants: juvenile; adult, reproductive and senescent. The habits of the studied plants are presented in Fig. 1. Considering the growth light conditions utilized in the current study (~175 μmol m^{-2} s^{-1} and 10 h photoperiod) the plants remained in the juvenile phase until around 2 weeks after germination, switched from adult phase to reproductive phase after the sixth week, and started to exhibit the first symptoms of senescence (increase of yellow-like spots) after 8 weeks (Supporting Information Table S1).

The dynamics of qPd, PSII yield and NPQ formation in the juvenile phase of plants are presented in Fig. 2. Here, qPd dropped below 0.98 when NPQ values were ~1.5 in 2-week-old plants, with NPQ conferring protection to significantly lower values (~0.5, estimated according with Fig. 2a) in 1-week-old plants (Fig. 2a,b; Tukey's test, $P < 0.05$). This reduction in qPd coincided with deviations between the calculated and observed PSII yield, as previously observed by Ruban & Belgio (2014), and is related to the photoinhibition of RCII. A further increase in light intensity caused more frequent occurrences of photoinhibition, depicted by grey and white diamonds (Fig. 2c,d). The amount of photoprotected and photoinhibited leaves at each of the light intensities was used to calculate the percentage of photoinhibition in leaf populations. One-week-old plants had 50% of the leaf population exhibiting photoinhibition (tolerated light) after exposure to a light intensity of ~70 μmol m^{-2} s^{-1} (Fig. 2e). Comparatively, the 2-week-old

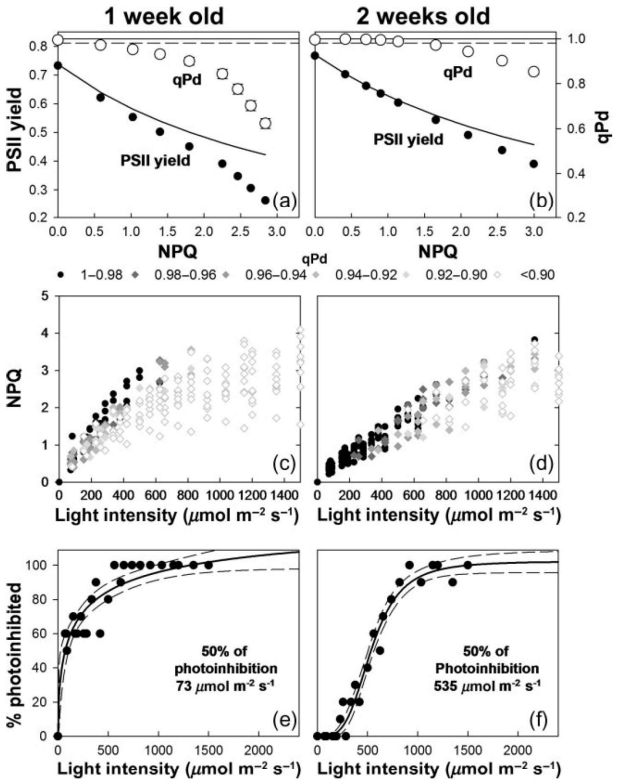

Figure 2. Photosynthetic dynamics of *Arabidopsis thaliana* plants at juvenile phase. Relationship between photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters measured on intact leaves from (a) 1- and (b) 2-week-old plants. The continuous line represents the theoretical yield, calculated by Eqn 1 of Materials and Methods. Error bars show the SEM ($n = 30$). Relationship between NPQ, light intensity and qPd parameters measured on leaves from (c) 1- and (d) 2-week-old plants. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. Percentage of photoinhibited leaves from (e) 1- and (f) 2-week-old plants. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

plants tolerated significant higher light (Z-test, $P < 0.05$) reaching ~300 μmol m^{-2} s^{-1} (Fig. 2f).

During the adult phase period (4- to 6-week-old plants), the dynamics of qPd and NPQ formation differed greatly from those exhibited by the young plants (Fig. 3; Supporting Information Figs S1 & S2). NPQ (6-week-old pNPQ = ~2.1) was protective to significantly higher light intensities (Fig. 3a,b; Tukey's test, $P < 0.05$) than in the juvenile phase. Furthermore, the amount of non-photoinhibited leaves in the vegetative growth stage was increased throughout the higher light intensities in comparison to the amount of non-photoinhibited leaves in the juvenile phase (Fig. 3c,d). As a consequence, the light intensity at which 50% of the leaves present photoinhibition was higher in plants during the adult phase in comparison to plants in the seedling stage (Z-test, $P < 0.05$). Plants of 4 weeks old presented

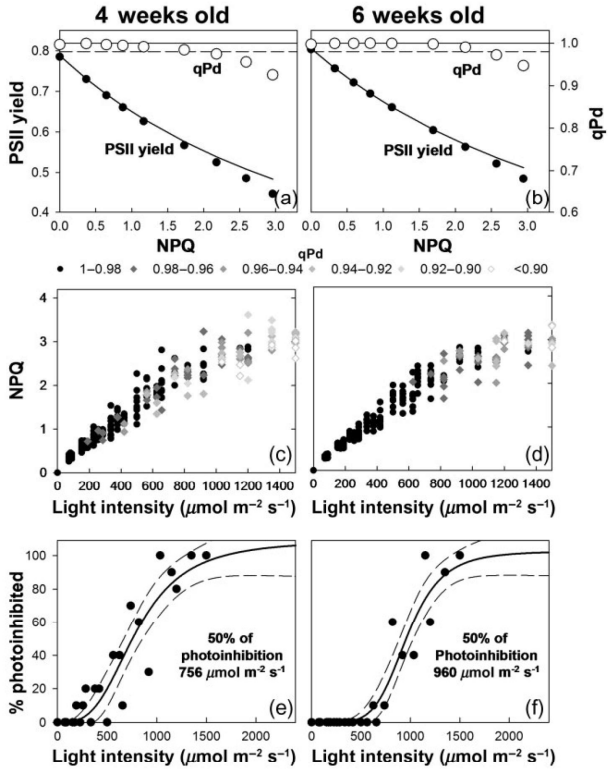

Figure 3. Photosynthetic dynamics of *Arabidopsis thaliana* plants at adult phase. Relationship between photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters measured on intact leaves from (a) 4- and (b) 6-week-old plants. The continuous line represents the theoretical yield, calculated by Eqn 1 of Materials and Methods. Error bars show the SEM ($n = 30$). Relationship between NPQ, light intensity and qPd parameters measured on leaves from (c) 4- and (d) 6-week-old plants. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. Percentage of photoinhibited leaves from (e) 4- and (f) 6-week-old plants. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

photoinhibition in 50% of the leaves at 756 μmol m^{-2} s^{-1} (Fig. 3e), with 6 week olds exhibiting tolerated light intensities up to 960 μmol m^{-2} s^{-1} (Fig. 3f).

Plants at the reproductive phase (7–8 weeks old) demonstrated a further increase in photoprotection as the dynamics in qPd decline and NPQ formation in comparison with both the juvenile and the adult phases (Fig. 4). The onset of photoinhibition was associated with NPQ values of ~2.5 and ~2.4 in plants 7 and 8 weeks old, respectively (Fig. 4a,b). These NPQ values significantly differed from those observed for 1, 2 and 4 weeks (Tukey's test, $P < 0.05$). The increase of non-photoinhibited leaves after exposure to very high light intensities is represented by the amount of closed circles in the graphs (Fig. 4c,d). As a consequence, the light intensities associated with 50% photoinhibition during the reproductive phase were as high as 1135 μmol m^{-2} s^{-1} in 7-week-old plants (Fig. 4e) and 1385 μmol m^{-2} s^{-1} in 8-week-old plants (Fig. 4f), which were the highest values observed in this study.

After 8 weeks, the onset of photoinhibition began to occur earlier. This is indicative of processes related to plant senescence taking place (Fig. 5; Supporting Information Figs S3–S5). The onset of photoinhibition in 11-week-old plants was associated with NPQ values of 1.7, and 0.8 in 13-week-old plants (Fig. 5a,b). pNPQ values in 13-week-old plants significantly differ from those observed in all other plant ages studied here (Tukey's test, $P < 0.05$). The plants in the senescent phase displayed reduced amounts of photoprotected leaves after exposure to higher light intensities, when compared with the plants in the vegetative growth stage, and especially at the reproductive phase (Fig. 5c,d; Z-test, $P < 0.05$). As a consequence, the intensities of light that induced 50% photoinhibition in plants at this phase was as

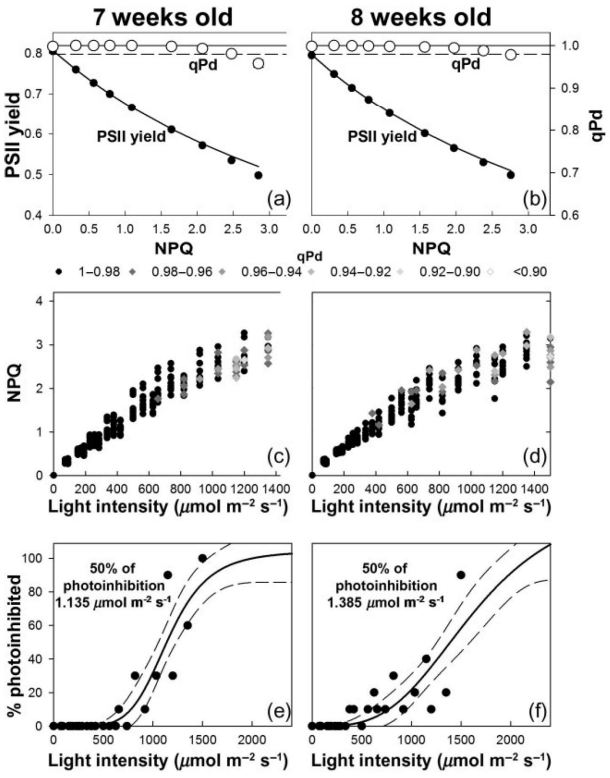

Figure 4. Photosynthetic dynamics of *Arabidopsis thaliana* plants at reproductive phase. Relationship between photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters measured on intact leaves from (a) 7- and (b) 8-week-old plants. The continuous line represents the theoretical yield, calculated by Eqn 1 of Materials and Methods. Error bars show the SEM ($n = 30$). Relationship between NPQ, light intensity and qPd parameters measured on leaves from (c) 7- and (d) 8-week-old plants. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. Percentage of photoinhibited leaves from (e) 7- and (f) 8-week-old plants. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

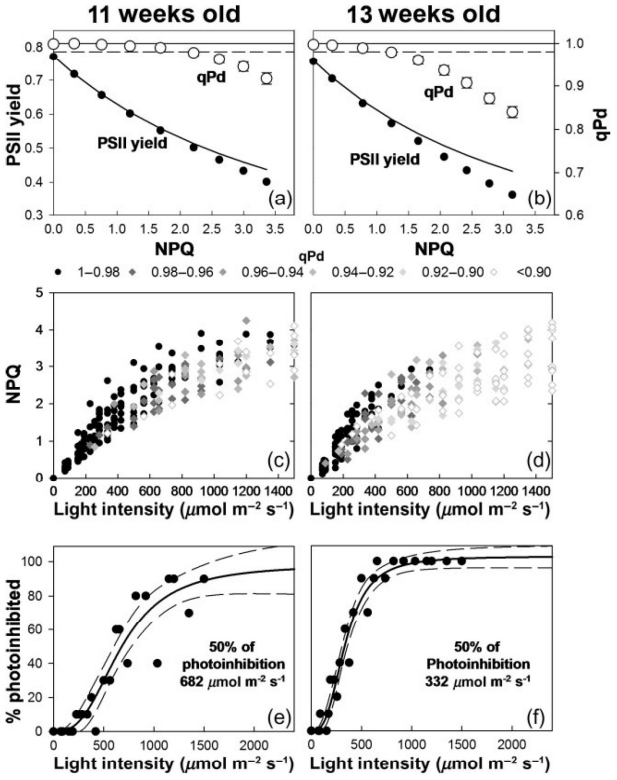

Figure 5. Photosynthetic dynamics of *Arabidopsis thaliana* plants at senescent phase. Relationship between photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters measured on intact leaves from (a) 11- and (b) 13-week-old plants. The continuous line represents the theoretical yield, calculated by Eqn 1 of Materials and Methods. Error bars show the SEM ($n = 30$). Relationship between NPQ, light intensity and qPd parameters measured on leaves from (c) 11- and (d) 13-week-old plants. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. Percentage of photoinhibited leaves from (e) 11- and (f) 13-week-old plants. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

low as 682 μmol m^{-2} s^{-1} in 11-week-old (Fig. 5e) and 332 μmol m^{-2} s^{-1} in 13-week-old plants (Fig. 5f).

pNPQ is a crucial factor of age-related light tolerance

The methodology for the quantification of photoinhibition employed here allowed the production of a time course of light tolerance of *A. thaliana* during its complete life cycle (Fig. 6a). The curve obtained here evidenced that the tolerated light intensity in *A. thaliana* plants increases between 1- and 5-week-old plants, reaching a maximum at 7–8 weeks after sowing. This is followed by a decrease with age, probably related with the rise in senescence processes. Moreover, the gradient obtained by the lowest pNPQ values at each light intensity revealed an increased efficiency of pNPQ for-

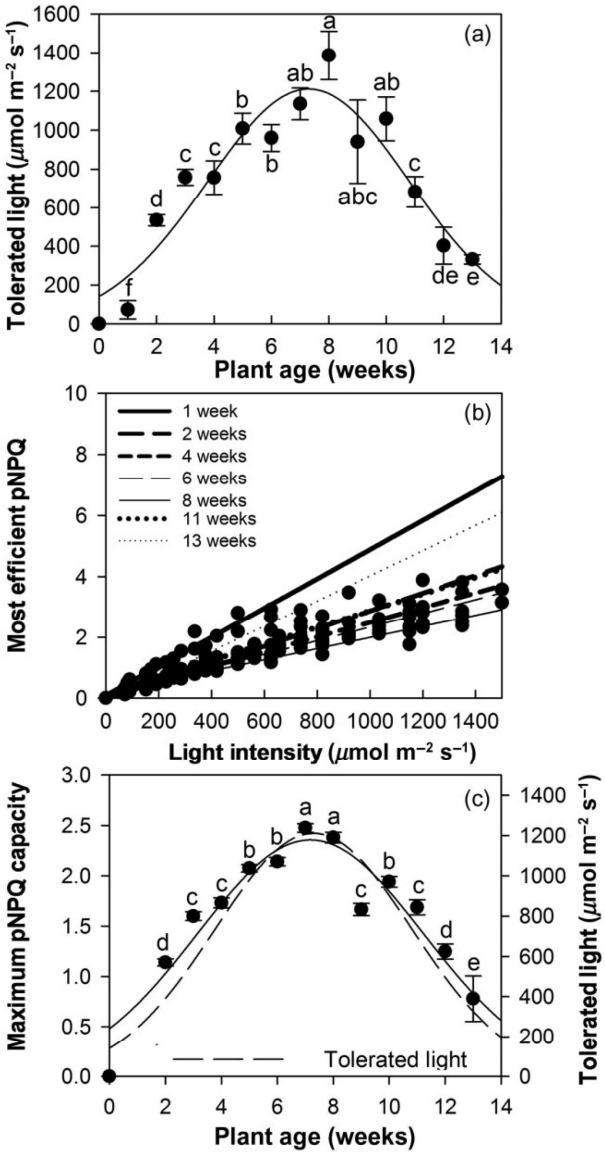

Figure 6. Light tolerance and protective NPQ (pNPQ) dynamics in *Arabidopsis thaliana* plants at 1 to 13 weeks old. (a) Light intensity which caused photoinhibition in 50% of leaf populations. The error bars represent the 95% confidence values plotted from regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$). Different letters represent a significant difference by Z-test, $P < 0.05$. The continuous line represents a regression fit curve (peak; Gaussian, three parameter $f = a \times \exp(-.5 \times ((x - x_0)/b)^2))$ plotted using SigmaPlot12 (Systat Software, Inc.). (b) Relationship between the most efficient pNPQ value (closed circles) and light intensity. The lines represent regression fit curves (polynomial; linear, $f = y0 + a^*x$). (c) Relationship between the maximum pNPQ capacity and plant age. The maximum pNPQ value was considered the first highest non-photochemical fluorescence quenching (NPQ) value which still protected 100% of RCII, calculated from the relationship between qPd and NPQ. Error bars show the SEM ($n = 30$) and different letters represent a significant difference by Tukey's test, $P < 0.05$. Continuous line represent regression fit curves (peak; Gaussian, three Parameter $f = a \times \exp(-.5 \times ((x - x_0)/b)^2))$. Dashed lines represent the tolerated light (50% of photoinhibited leaves).

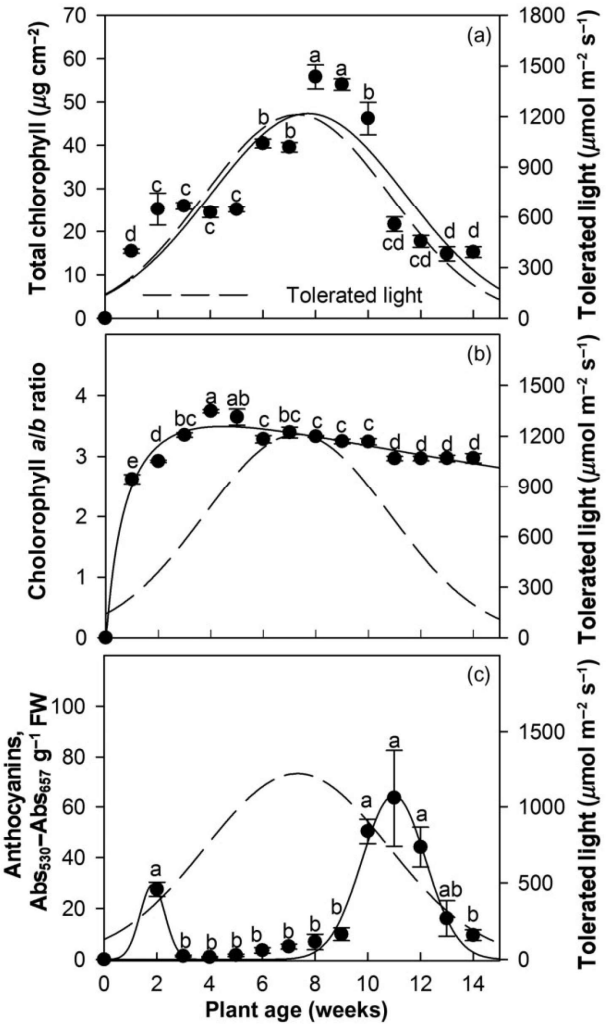

Figure 7. Pigment contents measured in leaves from *Arabidopsis thaliana* plants aged between 1–2 and 14 weeks old. (a) Total chlorophyll content; (b) chlorophyll *a*/*b* ratio and (c) total anthocyanin content. Error bars show the SEM ($n = 6$). Different letters represent significant difference by Tukey's test, $P < 0.05$. Total chlorophyll content was expressed as chlorophyll $a + b$ μg per leaf surface area (cm^{-2}). Anthocyanin content was expressed as the difference between the absorbance at 530 and at 657 nm per grams of fresh weight (g^{-1} FW).

mation from plants ageing between 1 and 8 weeks old, followed by a decline during the senescence stages (Fig. 6b). Significantly, the values of pNPQ as a function of plant age followed almost the same trend as the light tolerance data. The pNPQ values at juvenile and senescent phases significantly differ from those observed in the reproductive phase (Fig. 6c; Tukey's test, $P < 0.05$).

In order to test the role of the leaf pigments in age-dependent light tolerance, the contents of total chlorophyll and anthocyanins were quantified at each plant age (Fig. 7). The total chlorophyll content in leaves from plants aged between 1 and 14 weeks old varied with a similar trend to that observed in the tolerated light versus plant age curve (Fig. 7a). The total chlorophyll content presented an increase from 15.46 to 55.80 μg cm^{-2} in plants at 1 and 8 weeks old, respectively, followed by a significant decrease to 15.20 μg cm^{-2} at 14 weeks old (Tukey's test, $P < 0.05$). The chlorophyll *a*/*b* ratio, however, did not reveal a clear correlation with the light tolerance. The ratio increased in plants ageing from 1 to 4 weeks. This ranged from 2.61 at 1 week old, to 3.74 at 4 weeks old, where it reached a maximum, followed by a very gradual decrease to 2.97 at 14 weeks old (Fig. 7b; Tukey's test, $P < 0.05$). On the contrary, the dynamics of anthocyanin contents in leaves from different plant ages was completely different from the content of total chlorophyll and tolerated light versus plant age curves. The anthocyanin content was high at the juvenile phase (27.6 Abs530-Abs657 g FW^{-1}), very low in vegetative growth stages, accumulating gradually after 6 weeks (3.56 Abs530-Abs657 g FW^{-1}) before peaking between 10 and 12 weeks old (63.55 Abs530-Abs657 g FW^{-1} at 11 weeks old). This was followed by a decrease, exhibiting 9.54 Abs530-Abs657 g FW^{-1} of anthocyanins for 14-week-old plants (Fig. 7c; Tukey's test, $P < 0.05$). To ensure that the anthocyanin extracts were free from contaminants, a spectrophotometric scan was performed in extracts from 7-weeks-old plants (Supporting Information Fig. S6).

The ETR conforms with light tolerance curves

The F_v/F_m values increased between 1- and 7-week-old plants, from 0.737 to 0.806, reaching a maximum here. This was followed by a slight decrease after 8 weeks from 0.797 to 0.759 at 13 weeks (Fig. 8a). Differences were significant between 1 and 7 weeks old and between 7 and 13 weeks old (Tukey's test, $P < 0.05$). The ETR values were measured over the course of the procedure at each of the light intensities utilized to obtain the qPd values (Supporting Information Fig. S7). Furthermore, the ETR values at 500 μmol m^{-2} s^{-1} of light intensity were used to compare the performance of different plant ages (Fig. 8b). The ETR varied in a very similar pattern from that observed in the light tolerance curves. The ETR curve consisted of a first phase of significant increase from 1 to 8 weeks old (ranging from 25.6 to 79.4 μmol m^{-2} s^{-1}), reaching a maximum plateau at 8 weeks. This was followed by a significant decrease phase ranging from 61.2 μmol m^{-2} s^{-1} at 9 weeks old to 24.68 μmol m^{-2} s^{-1} at 13 week old (Tukey's test, $P < 0.05$).

Light tolerance of different aged leaves of the same plant

Aiming to verify whether differences of photoprotection exist in leaves with different physiological ages in the same rosette of a given plant age, intermediate and internal leaves from plants ageing 8 weeks old were also tested (Supporting Information Figs S8 & S9). The external leaves from rosettes at 8 weeks old were already tested as previously mentioned (Fig. 4). The light intensity related to 50% photoinhibition presented several differences amongst the different physiological stages of leaves in the same rosette (Fig. 9). Whilst the

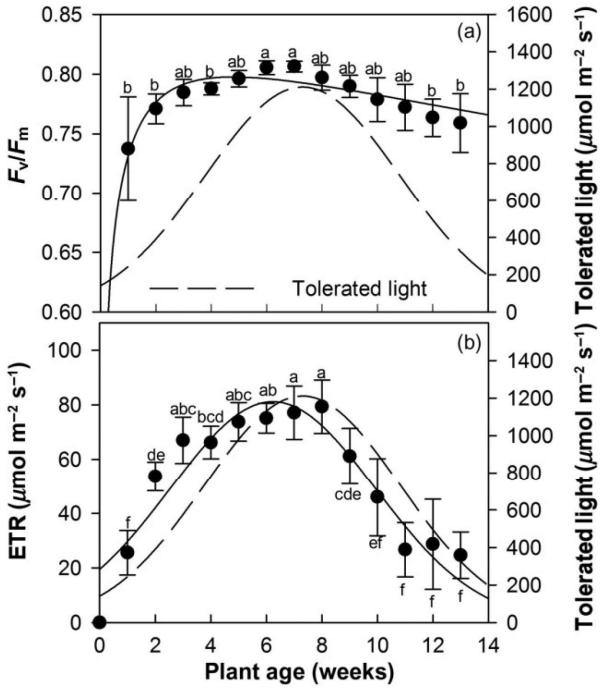

Figure 8. Relationship between chlorophyll *a* parameters and light intensity related to photoinhibition in 50% of leaf populations of *Arabidopsis thaliana* plants aged between 1 and 13 weeks. (a) Maximum quantum yield [F_v/F_m] versus plant age and (b) Electron transport rate (ETR). The values of ETR were collected at 500 μmol m^{-2} s^{-1} of actinic light intensity from light curves ranging from 0 to 1500 μmol m^{-2} s^{-1}. Error bars show the SD ($n = 30$). Different letters represent significant differences by Tukey's test, $P < 0.05$.

external leaves from plants at 8 weeks old presented a tolerated light intensity equal to 1385 μmol m^{-2} s^{-1}, the intermediate and internal leaves presented significantly lower tolerance, respectively 856 and 643 μmol m^{-2} s^{-1} (Fig. 9a; *Z*-test, $P < 0.05$). The F_v/F_m values from internal leaves, however, displayed no significant changes between the external and intermediate leaves (Fig. 9b; Tukey's test, $P > 0.05$).

Different ROS accumulation during ontogenetic phases is in agreement with anthocyanin content

In order to test the hypothesis that the trend observed for anthocyanin content during plant ontogenesis is related to antioxidative roles, the *in situ* accumulation of H_2O_2 and the lipid peroxidation/MDA formation (TBARS) were performed. Higher amounts of H_2O_2 were observed in leaves from plants at juvenile and senescent phases in comparison with leaves from plants at adult and reproductive phases (Fig. 10a). The H_2O_2 accumulation was detected by the appearance of brown spots especially at the superior part of the leaves and veins. Corroborating the higher amount of H_2O_2 in juvenile and senescent phased leaves, the lipid peroxidation evaluated biochemically by the MDA accumulation (TBARS) was also higher in these two phases in comparison with the data for adult and reproductive phase leaves (Fig. 10b; Tukey's test, $P > 0.05$).

DISCUSSION

Plant ontogenesis is affected by many diverse variables. These can be both endogenous, as a result of genomic programming in the course of evolution, and external, affected by abiotic and biotic factors, especially varying light intensities, temperature, nutrient and water availability (Tichá 1985; Tsukaya 2013). It is perhaps due to the inherent difficulty of assessing the effects of isolated stresses in this multicomplex process, that even after decades of research of plant ontogenesis, several questions remain unanswered (Tichá 1985; Schurr *et al.* 2006; Tsukaya 2013). Numerous studies have provided insights about the effects of ontogenesis on different physiological processes such as leaf growth (Avery 1933; Milthorpe 1959; Hopkinson 1964), leaf development (van Lijsebettens & Clarke 1998; Tsukaya 2013) and photosynthesis (Biswal & Mohanty 1976; Čatský & Tichá 1979; Siffel *et al.* 1993; Thiele *et al.* 1997; Bondada & Oosterhuis 1998; Nadeau & Sack 2002; Walter *et al.* 2004; Radochová & Tichá 2008; Liu *et al.* 2009; Nath *et al.* 2013). However, the required light intensity that is high enough to produce photoinhibition in leaves during different ontogenetic stages remains poorly understood.

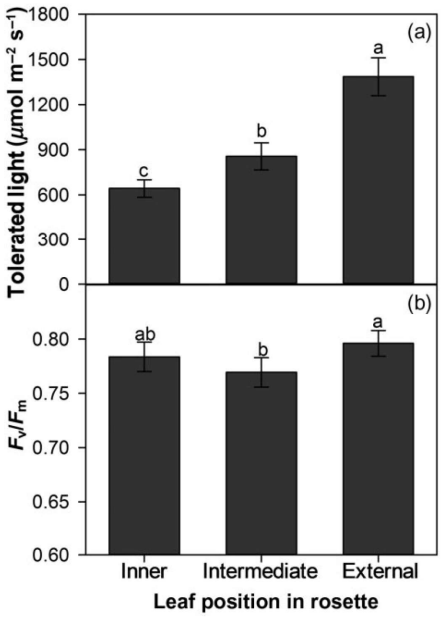

Figure 9. Light tolerance and protective NPQ (pNPQ) dynamics of inner, intermediate and external leaves from 8-week-old *Arabidopsis thaliana* plants. (a) Light intensity which caused photoinhibition in 50% of leaf populations. The error bars represent the 95% confidence values plotted from regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) using SigmaPlot12 (Systat Software, Inc.). Different letters represent a significant difference by *Z*-test, $P < 0.05$. (b) Maximum quantum yield [F_v/F_m]. Error bars show the SD ($n = 30$). Different letters represent significant difference by Tukey's test, $P < 0.05$.

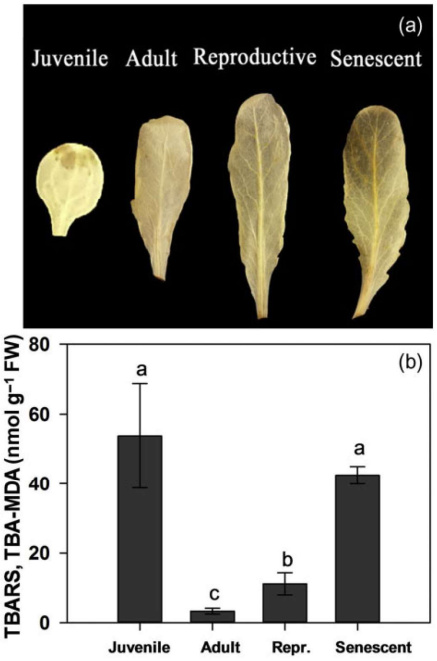

Figure 10. Age-dependent reactive oxygen species (ROS) accumulation indicators in leaves of *Arabidopsis thaliana*. (a) *In situ* H_2O_2 detection assessed by 3,3′-diaminobenzidine (DAB) staining. (b) Lipid peroxidation, as indicated by thiobarbituric acid-reactive substances (TBARS). Error bars show the standard deviation ($n = 3$). Different letters represent significant differences by Tukey's test, $P < 0.05$. The leaves used in the assays were always the most externally positioned in the rosette at respective ontogenetic phases: juvenile (2 weeks), adult (5 weeks), reproductive (8 weeks) and senescent (11 weeks).

Here we proposed the use of the recently described methodology for the determination of qPd and pNPQ (Ruban & Murchie 2012) to assess the dynamics of the photosynthetic response to light fluctuations during ontogenesis. It is clear that, similar to previous reports on several species, which used F_v/F_m (Siffel *et al.* 1993; Barker *et al.* 1997; la Porta *et al.* 2006; Liu *et al.* 2009), Hill reaction activity (Kutík *et al.* 2000) and CO_2 assimilation parameters (Šesták & Čatský 1962; Oquist *et al.* 1982; la Porta *et al.* 2006; Radochová & Tichá 2008), the amount of light tolerated by leaves was found to be also dependent upon plant age. Furthermore, this consists of a well-defined dynamic divided into three phases: a first phase tolerance increase, a plateau phase at maximum tolerance and a tolerance decrease phase. This last phase is probably related to senescence processes (Biswal & Mohanty 1976; Bondada & Oosterhuis 1998; Nath *et al.* 2013). The patterns observed in photosynthesis during ontogenesis might be a consequence of energetic demands from metabolic changes related to seedling growth, reproductive processes and cell death.

Using the methodology described by Ruban & Murchie (2012), it was possible for the first time to determine here the amount of pNPQ formed in a range of light intensities from 0 to 1500 μmol m^{-2} s^{-1}, throughout different phases of the *Arabidopsis* ontogeny. The pNPQ amplitude displayed a very similar trend to age-dependent light tolerance curves, where the maximum pNPQ values were observed in the reproductive phase, with lower values during juvenile and senescent phases. Indeed, plants aged 8 weeks old (reproductive phase) were able to tolerate light intensities ~2.6 times higher than 2-week-old plants (juvenile phase), and ~4.2 times higher than 13-week-old plants (senescent phase). Considering these remarkable differences, it is reasonable to deduce that the age-dependent pNPQ gradient should have contributed to this process. Interestingly, our previous studies on *Arabidopsis* *–npq4* (lacking PsbS protein), *–L17* (an overexpresser of PsbS protein) and *–npq1* (unable to convert violaxanthin to zeaxanthin) have exhibited the same gradients of pNPQ formation in comparison to the wild type (Ruban & Belgio 2014; Ware *et al.* 2015). Thus, two important ideas could be raised here: (1) pNPQ formation is age dependent and (2) pNPQ formation is crucial to light tolerance of *Arabidopsis* plants during ontogenesis.

Indeed, plants with the same age exhibited differences between the internal, intermediate and external leaves concerning the tolerated light intensity. The inner leaves were more sensitive to photoinhibition than the intermediate and the external leaves. This is in agreement with the age-dependent light tolerance verified in external leaves during plant ontogenesis. Thus photoinhibition in *Arabidopsis* plants is dependent not only on plant age but also on leaf age. Moreover, besides the differences of light intensity related to 50% of photoinhibition between different leaf ages, no significant differences in F_v/F_m were observed between leaves at different positions in the same rosette. This would indicate that the maximum photochemical yield of RCII does not change dramatically, yet the protective capacity of NPQ requires some sort of evolution. These data corroborate the efficiency of the qPd parameter in quantifying photoinhibition, as well as reinforce its crucial roles in the light tolerance of plants.

Interestingly, the juvenile and the senescent plants studied here presented the higher values of maximum NPQ at the lower light intensities, but as they became photoinhibited earlier, NPQ was not protective. Higher maximum NPQ values in young compared to mature leaves have been reported in several plant species (Dodd *et al.* 1998; Manetas *et al.* 2002; Liu *et al.* 2009). The sensitivity to photoinhibition by young plants in comparison to mature ones is, however, believed to be species dependent (Krause *et al.* 1995; Dodd *et al.* 1998; Manetas *et al.* 2002; Choinski Jr. *et al.* 2003; Liu *et al.* 2009). Some species seem to exhibit higher photoinhibition at juvenile phases in comparison to maturity, as reported for *Castilla elastica*, *Ficus insipida*, *Antirrhoea trichantha* and *Anacardium excelsum* (Krause *et al.* 1995). In contrast, other species present higher photoinhibition at the mature phase, as reported for *Rosa* sp. and *Ricinus communis* (Manetas *et al.* 2002). Besides the different behaviours between species, the common conclusion is that phototolerance is a consequence of activity of antioxidative enzymes, pigment composition and total NPQ formation.

Several different metabolic processes take place in plants at the reproductive phase, such as flowering (Chailakhyan 1968) and seed formation (Goldberg *et al.* 1994), which, in turn, could act as an energy sink that would be favourable to light tolerance. In principle, the estimate of the non-cyclic ETR through PSII could provide some clues on the amount of light energy that has been utilized by the plants (Baker 2008), in this specific case, at different ontogenetic phases. Indeed, in the plants studied here, the ETR values varied through the different plant ages in a similar trend to the tolerated light intensities (50% of photoinhibition). This means that plants at the reproductive phase were more efficient in transporting electrons in the thylakoid membranes for the formation of ATP and NADPH. A combination between more efficient pNPQ formation and ETR should have ensured *Arabidopsis* plants at the reproductive phase have a better ability to cope with high light than the juvenile and senescent phases. However, care is needed when using the ETR parameter to compare plants at different ontogenetic phases, as variations in the leaf thickness and chlorophyll content are common (Avery 1933; Šesták & Čatský 1962; Babani & Lichtenthaler 1996; la Porta *et al.* 2006; Liu *et al.* 2009). Moreover, ontogenetic changes in PSII/PSI ratio between the juvenile, mature and senescent plants could also interfere in the ETR calculus (Baker 2008). These results could lead to a miscalculation of the true increase in ETR capacity of mature plants.

Light tolerance associated with *Arabidopsis* ontogenesis also presented a similar trend to the total chlorophyll content. Indeed, the roles of chlorophyll molecules in antennae function and composition have been known for a long time, contributing to both the capture and dissipation of energy (Peter & Thornber 1991; Bassi & Dainese 1992; Ruban *et al.* 1999; Hogewoning *et al.* 2012; van Amerongen & Croce 2013). The age-dependent pattern in chlorophyll content exhibited here was also registered in several plant species, such as *Nicotiana sanderae* (Šesták & Čatský 1962) and *Cupressus sempervirens* (la Porta *et al.* 2006). This pattern is also associated with CO_2 assimilation rates (Šesták & Čatský 1962; Babani & Lichtenthaler 1996; la Porta *et al.* 2006).

Indeed, several cellular and anatomical changes occur during the leaf expansion and subsequent senescence. The size of thylakoid membranes is known to increase in mature plants compared to juveniles, whilst the stroma size decreases (Kutík *et al.* 2000). During senescence, the breakdown of thylakoid membranes is accompanied by the increase of plastoglobuli formation (Kutík *et al.* 2000). In parallel, the leaf thickness is also increased during the transition from juvenile to mature phases (Avery 1933). The palisade cell expansion and development in the intercellular spaces of the spongy parenchyma are believed to occupy about one-third of the mature leaf length. This contributes to the leaf thickness increase during this period (Tichá 1985). Moreover, sun leaves are known to present increased chlorophyll content related to increased thickness of palisade tissue in comparison to shade-adapted leaves (Cui *et al.* 1991). Since the chlorophyll content is related to leaf thickness, chloroplast expansion and thylakoid size, we propose that the similar trend between chlorophyll content and age-dependent light tolerance was possibly a consequence of the efficiency of light screening during the different ontogenetic phases. These screening mechanisms should have acted in parallel with pNPQ in the protection of plant leaves against photoinhibition, to which plants at juvenile and senescent phases were more sensitive.

Nath *et al.* (2013), working with *Arabidopsis* leaves during the senescent phase, observed decreases in F_v/F_m, NPQ, ETR, total chlorophyll and D1 protein amount, but the chlorophyll *a/b* ratios and the quantity of antenna proteins were more stable. These results are in conformity with the data presented here on age-dependent light tolerance that presented a similar trend to the amount of total chlorophyll and the formation of pNPQ. Moreover, during senescence, the loss of chlorophyll, as well as changes in chloroplast ultrastructure (Bondada & Oosterhuis 1998), and possibly the accumulation of ROS could have contributed to the decrease in the light tolerance observed. Indeed, during senescence, several changes in the cellular metabolism take place. Increases in concentrations of H_2O_2 and other ROS, degradation of carbohydrate reserves, proteins, lipid membranes and chlorophyll molecules have been reported frequently (Biswal & Mohanty 1976; Munné-Bosch & Alegre 2002; Hörtensteiner 2006; Zimmermann *et al.* 2006; Sabater & Martín 2013).

Interestingly, the dynamics of anthocyanin content during ontogenesis was completely different from that of the total chlorophyll and the light tolerance curves. The anthocyanins were accumulated only in the initial and final stages of the ontogenetic cycle studied here, reaching the maximum values when the plants were more sensitive to the high light intensities. This pattern was also previously reported for six different species (Liu *et al.* 2009). Despite the described roles of anthocyanins in UV-light screening (Takahashi & Badger 2011), the accumulation of these molecules here apparently did not contribute crucially to an increase in the high light tolerance during ontogenesis. Indeed, the major capability of light absorption by anthocyanins occurs at UV range wavelength. It means that these molecules are perhaps able to contribute only in a minor extension to the screening of visible light (especially around 550 nm); therefore, the fraction of light related to Chl excitation should not be greatly affected. Moreover, considering the well-known roles of anthocyanins acting as antioxidants in plants (Zeng *et al.* 2010), it is reasonable to argue that the juvenile and senescent anthocyanin accumulation observed here should be related to their antioxidant function, rather than light filtration effect (Krause *et al.* 1995; Dodd *et al.* 1998; Liu *et al.* 2009; Zhang *et al.* 2014). Accordingly, with this hypothesis, the juvenile and senescent leaves exhibited here the highest H_2O_2 accumulation (evaluated by *in situ* detection) and oxidative stress (evaluated by TBARS).

Taken together, our data suggest that the *A. thaliana* plants exhibit an efficient dynamic associated with the regulation of photosynthesis in response to fast fluctuating light exposure during ontogenesis. The amount of light tolerated by a given leaf was quantified here, and our results indicate that the

tolerated light intensities of leaves are strongly dependent on plant age and the leaf developmental stage. The light intensity tolerated increased from the first week after sowing until 8 weeks, followed by a decrease to 13 weeks. This trend was similar to the observed total chlorophyll content, ETR and pNPQ formation. This was completely different from anthocyanin accumulation. Additionally, leaves with different physiological ages in the same rosette presented differences in pNPQ in accordance with tolerated light intensities. Thus, the pNPQ formation is age dependent and in parallel with ETR and pigment light screening mechanisms; it is a crucial factor in defining the ontogenetic tolerance of light exposure in *A. thaliana* plants. Moreover, the successful use of this new methodology for quantifying the photosynthetic dynamics and light tolerance during *Arabidopsis* ontogenesis reveals the great potential of the qPd parameter as an efficient tool in the quantification of early symptoms of photoinhibition at different physiological conditions.

ACKNOWLEDGMENTS

AVR would like to thank UK BBSRC and The Leverhulme Trust for the financial support. Fabricio E.L. Carvalho is supported by the Science Without Boarders program (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq – Brazil).

REFERENCES

Adams I.I.I.W.W., Zarter C.R., Mueh K.E., Amiard V. & Demmig-Adams B. (2006) Energy dissipation and photoinhibition: a continuum of photoprotection. In *Photoprotection, Photoinhibition, Gene Regulation, and Environment* (eds B. Demmig-Adams, W.W. Adams & A.K. Mattoo), pp. 49–64. Springer, Dordrecht.

Ahn T.K., Avenson T.J., Ballottari M., Cheng Y.-C., Niyogi K.K., Bassi R. & Fleming G.R. (2008) Architecture of a charge-transfer state regulating light harvesting in a plant antenna protein. *Science* **320,** 794–797.

Amasino R. (2010) Seasonal and developmental timing of flowering. *The Plant Journal* **61,** 1001–1013.

Arnon D.I., Allen M.B. & Whatley F.R. (1954) Photosynthesis by isolated chloroplasts. *Nature* **174,** 394–396.

Aro E.-M., McCaffery S. & Anderson J.M. (1993) Photoinhibition and D1 protein degradation in peas acclimated to different growth irradiances. *Plant Physiology* **130,** 835–843.

Avery G.S. (1933) Structure and development of the tobacco leaf. *American Journal of Botany* **20,** 565–592.

Babani F. & Lichtenthaler H.K. (1996) Light-induced and age-dependent development of chloroplasts in etiolated barley leaves as visualized by determination of photosynthetic pigments, CO_2 assimilation rates and different kinds of chlorophyll fluorescence ratios. *Journal of Plant Physiology* **148,** 555–566.

Bailey S., Walters R.G., Jansson S. & Horton P. (2001) Acclimation of *Arabidopsis thaliana* to the light environment: the existence of separate low light and high light responses. *Planta* **213,** 794–801.

Baker N.R. (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annual Review of Plant Biology* **59,** 89–113.

Ballottari M., Dall'Osto L., Morosinotto T. & Bassi R. (2007) Contrasting behavior of higher plant photosystem I and II antenna systems during acclimation. *The Journal of Biological Chemistry* **282,** 8947–8958.

Barber J. (2002) Photosystem II: a multisubunit membrane protein that oxidises water. *Current Opinion in Structural Biology* **12,** 523–530.

Barker D.H., Seaton G.G.R. & Robinson S.A. (1997) Internal and external photoprotection in developing leaves of the CAM plant *Cotyledon orbiculata*. *Plant, Cell & Environment* **20,** 617–624.

Bassi R. & Dainese P. (1992) A supramolecular light harvesting complex from chloroplast photosystem II membranes. *European Journal of Biochemistry* **204,** 317–326.

Bassi R., Hoyer-Hansen G., Barbato R., Giacometti G.M. & Simpson D.J. (1987) Chlorophyll-proteins of the photosystem II antenna system. *The Journal of Biological Chemistry* **262,** 13333–13341.

Bellafiore S., Barneche F., Peltier G. & Rochaix J.-D. (2005) State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. *Nature* **433,** 892–895.

Betterle N., Ballottari M., Zorzan S., de Bianchi S., Cazzaniga S., Dall'Osto L., . . . Bassi R. (2009) Light-induced dissociation of an antenna hetero-oligomer is needed for non-photochemical quenching induction. *The Journal of Biological Chemistry* **284,** 15255–15266.

Biswal U. & Mohanty P. (1976) Aging induced changes in photosynthetic electron transport of detached barley leaves. *Plant & Cell Physiology* **17,** 323–331.

Boekema E.J., Hankamer B., Bald D., Kruip J., Nield J., Boonstra A.F., . . . Rögner M. (1995) Supramolecular structure of the photosystem II complex from green plants and cyanobacteria. *Proceedings of the National Academy of Sciences USA* **92,** 175–179.

Bondada B. & Oosterhuis D. (1998) Decline in photosynthesis as related to alterations in chloroplast ultrastructure of a cotton leaf during ontogeny. *Photosynthetica* **35,** 467–471.

Borsani O., Valpuesta V. & Botella M. (2001) Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in Arabidopsis seedlings. *Plant Physiology* **126,** 1024–1030.

Boyes D.C., Zayed A.M., Ascenzi R., McCaskill A.J., Hoffman N.E., Davis K.R. & Görlach J. (2001) Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. *The Plant Cell* **13,** 1499–1510.

Briantais J.-M., Vemotte C., Picaud M. & Krause G.H. (1979) A quantitative study of the slow decline of chlorophyll *a* fluorescence in isolated chloroplasts. *Biochimica et Biophyssica Acta* **548,** 128–138.

Cakmak I. & Horst W.J. (1991) Effect of aluminum on lipid peroxidation, superoxide-dismutase, catalase and peroxidase activities in root-tips of soybean (*Glycine max*). *Physiologia Plantarum* **83,** 463–468.

Chailakhyan M. (1968) Internal factors of plant flowering. *Annual Review of Plant Physiology* **19,** 1–37.

Choinski Jr. J.S., Ralph P.A. & Eamus D.A. (2003) Changes in photosynthesis during leaf expansion in *Corymbia gummifera*. *Australian Journal of Botany* **51,** 111–118.

Cui M., Vogelmann T.C. & Smith W.K. (1991) Chlorophyll and light gradients in sun and shade leaves of *Spinacia oleracea*. *Plant, Cell and Environment* **14,** 493–500.

Čatský J. & Tichá I. (1979) CO_2 compensation concentration in bean leaves: Effect of photon flux density and leaf age. *Biologia Plantarum* **21,** 361–364.

Demmig-Adams B. & Adams W.W. III. (1992) Photoprotection and other responses of plants to high light stress. *Annual Review of Plant Physiology and Plant Molecular Biology* **43,** 599–626.

Dodd I.C., Critchley C., Woodall G.S. & Stewart G.R. (1998) Photoinhibition in differently coloured juvenile leaves of Syzygium species. *Journal of Experimental Botany* **49,** 1437–1445.

Färber A., Young A.J., Ruban A.V., Horton P. & Jahns P. (1997) Dynamics of xanthophyll cycle activity in different antenna subcomplexes in the photosynthetic membranes of higher plants. *Plant Physiology* **115,** 1609–1618.

Foyer C.H. & Shigeoka S. (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiology* **155,** 93–100.

Funk C., Schroder W.P., Napiwotzki A., Tjus S.E., Renger S.G. & Anderson B. (1995) The PSII-S Protein of Higher Plants: A New Type of Pigment-Binding Protein? *Biochemistry* **34,** 11133–11141.

Goldberg R., de Paiva G. & Yadegari R. (1994) Plant embryogenesis: zygote to seed. *Science* **266,** 605–614.

Hideg E., Kós P.B. & Schreiber U. (2008) Imaging of NPQ and ROS formation in tobacco leaves: heat inactivation of the water-water cycle prevents down-regulation of PSII. *Plant & Cell Physiology* **49,** 1879–1886.

Hill R. & Bendall F. (1960) Function of the two cytochrome components in chloroplasts: a working hypothesis. *Nature* **186,** 136–137.

Hogewoning S.W., Wientjes E., Douwstra P., Trouwborst G., van Ieperen W., Croce R. & Harbinson J. (2012) Photosynthetic quantum yield dynamics: from photosystems to leaves. *The Plant Cell* **24,** 1921–1935.

Hopkinson J. (1964) Studies on the expansion of the leaf surface IV. The carbon and phosphorus economy of a leaf. *Journal of Experimental Botany* **15,** 125–137.

Horton P. & Ruban A.V. (1992) Regulation of photosystem II. *Photosynthesis Research* **34,** 375–385.

Horton P., Ruban A.V. & Walters R.G. (1996) Regulation of light harvesting in green plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47,** 655–684.

Hörtensteiner S. (2006) Chlorophyll degradation during senescence. *Annual Review of Plant Biology* **57,** 55–77.

Ivanov A.G., Rosso D., Savitch L.V., Stachula P., Rosembert M., Oquist G. & Hüner N.P. (2012) Implications of alternative electron sinks in increased resistance of PSII and PSI photochemistry to high light stress in cold-acclimated *Arabidopsis thaliana*. *Photosynthesis Research* **113,** 191–206.

Jahns P. & Holzwarth A.R. (2012) The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. *Biochimica et Biophysica Acta* **1817,** 182–193.

Johnson M.P. & Ruban A.V. (2011) Restoration of rapidly reversible photoprotective energy dissipation in the absence of psbs protein by enhanced ΔpH. *The Journal of Biological Chemistry* **286,** 19973–19981.

Kerstetter R. & Poethig R. (1998) The specification of leaf identity during shoot development. *Annual Review of Cell and Developmental Biology* **14,** 373–398.

Kouřil R., Wientjes E., Bultema J.B., Croce R. & Boekema E.J. (2013) High-light vs. low-light: effect of light acclimation on photosystem II composition and organization in *Arabidopsis thaliana*. *Biochimica et Biophysica Acta* **1827,** 411–419.

Krause G.H. (1974) Changes in chlorophyll fluorescence in relation to light-dependent cation transfer across thylakoid membranes. *Biochimica et Biophysica Acta* **333,** 301–313.

Krause G.H., Virgo A. & Winter K. (1995) High susceptibility to photoinhibition of young leaves of tropical forest trees. *Planta* **197,** 583–591.

Kutík J., Kočová M., Holá D. & Körnerová M. (2000) The development of chloroplast ultrastructure and hill reaction activity during leaf ontogeny in different maize (*Zea mays* L.) genotypes. *Photosynthetica* **36,** 497–507.

Li X.P., Björkman O., Shih C., Grossman A.R., Rosenquist M., Jansson S. & Niyogi K.K. (2000) A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature* **403,** 391–395.

Li X.P., Muller-Moule P., Gilmore A.M. & Niyogi K.K. (2002) PsbS-dependent enhancement of feedback de-excitation protects photosystem II from photoinhibition. *Proceedings of the National Academy of Sciences USA* **99,** 15222–15227.

Liu N., Lin Z.-F., van Devender A., Lin G.-Z., Peng C.-L. & Pan X.-P. (2009) Spectral reflectance indices and pigment functions during leaf ontogenesis in six subtropical landscape plants. *Plant Growth Regulation* **58,** 73–84.

Manetas Y., Drinia A. & Petropoulou Y. (2002) High contents of anthocyanins in young leaves are correlated with low pools of xanthophyll cycle components and low risk of photoinhibition. *Photosynthetica* **40,** 349–354.

Matile P., Schellenberg M. & Peisker C. (1992) Production and release of a chlorophyll catabolite in isolated senescent chloroplasts. *Planta* **187,** 230–235.

Matsubara S. & Chow W. (2004) Populations of photoinhibited photosystem II reaction centers characterized by chlorophyll *a* fluorescence lifetime *in vivo*. *Proceedings of the National Academy of Sciences USA* **101,** 18234–18239.

Milthorpe F. (1959) Studies on the expansion of the leaf surface I. The influence of temperature. *Journal of Experimental Botany* **10,** 233–249.

Munné-Bosch S. & Alegre L. (2002) Plant aging increases oxidative stress in chloroplasts. *Planta* **214,** 608–615.

Murata N. (1969) Control of excitation transfer in photosynthesis. I. Light-induced change of chlorophyll *a* fluorescence in *Porphyridium cruentum*. *Biochimica et Biophysica Acta* **172,** 242–251.

Nadeau J. & Sack F. (2002) Control of stomatal distribution on the Arabidopsis leaf surface. *Science* **296,** 1697–1700.

Nath K., Phee B.-K., Jeong S., Lee S.Y., Tateno Y. & Allakhverdiev S.I. (2013) Age-dependent changes in the functions and compositions of photosynthetic complexes in the thylakoid membranes of *Arabidopsis thaliana*. *Photosynthesis Research* **117,** 547–556.

Neff M. & Chory J. (1998) Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during Arabidopsis development. *Plant Physiology* **118,** 27–36.

Nilkens M., Kress E., Lambrev P., Miloslavina Y., Müller M., Holzwarth A.R. & Jahns P. (2010) Identification of a slowly inducible zeaxanthin-dependent component of non-photochemical quenching of chlorophyll fluorescence generated under steady-state conditions in *Arabidopsis*. *Biochimica et Biophysica Acta* **1797,** 466–475.

Nishiyama Y., Allakhverdiev S.I. & Murata N. (2011) Protein synthesis is the primary target of reactive oxygen species in the photoinhibition of photosystem II. *Physiologia Plantarum* **142,** 35–46.

Niyogi K.K., Li X.-P., Rosenberg V. & Jung H.-S. (2005) Is PsbS the site of non-photochemical quenching in photosynthesis? *Journal of Experimental Botany* **56,** 375–382.

Noodén L.D. (1988) The phenomenon of senescence and aging. In *Senescence and Aging in Plants* (eds L.D. Noodén & A.C. Leopold), pp. 1–50. Academic Press, San Diego.

Oquist G., Brunes L. & Hallgren J.-E. (1982) Photosynthetic efficiency during ontogenesis of leaves of *Betula pendula*. *Plant, Cell and Environment* **5,** 17–21.

Oxborough K. & Baker N.R. (1997) Resolving chlorophyll *a* fluorescence of photosynthetic efficiency into photochemical components – calculation of qP and Fv′/Fm′ without measuring Fo. *Photosynthesis Research* **54,** 135–142.

Paul M.J. & Foyer C.H. (2001) Sink regulation of photosynthesis. *Journal of Experimental Botany* **52,** 1383–1400.

Peter G. & Thornber J. (1991) Biochemical composition and organization of higher plant photosystem II light-harvesting pigment-proteins. *Journal of Biological Chemistry* **266,** 16745–16754.

Porra R., Thompson W. & Kriedemann P. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents. *Biochimica et Biophysica Acta* **975,** 384–394.

la Porta N., Bertamini M., Nedunchezhian N. & Muthuchelian K. (2006) Photosynthetic changes that occur during aging of cypress (*Cupressus sempervirens* L.) needles. *Photosynthetica* **44,** 555–560.

Radochová B. & Tichá I. (2008) Excess irradiance causes early symptoms of senescence during leaf expansion in photoautotrophically in vitro grown tobacco plants. *Photosynthetica* **46,** 471–475.

Raven J.A. (1984) Physical correlates of the morphology of early vascular plants. *Botanical Journal of the Linnean Society* **88,** 105–126.

Ruban A.V. (2015) Evolution under the sun: optimizing light harvesting in photosynthesis. *Journal of Experimental Botany* **66,** 7–23.

Ruban A.V. & Belgio E. (2014) The relationship between maximum tolerated light intensity and photoprotective energy dissipation in the photosynthetic antenna: chloroplast gains and losses. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **369,** 20130222.

Ruban A.V. & Murchie E.H. (2012) Assessing the photoprotective effectiveness of non-photochemical chlorophyll fluorescence quenching: a new approach. *Biochimica et Biophysica Acta* **1817,** 977–982.

Ruban A.V., Lee P.J., Wentworth M., Young A.J. & Horton P. (1999) Determination of the stoichiometry and strength of binding of xanthophylls to the photosystem II light harvesting complexes. *Journal of Biological Chemistry* **274,** 10458–10465.

Ruban A.V., Berera R., Ilioaia C., van Stokkum I.H.M., Kennis J.T.M., Pascal A.A., ... van Grondelle R. (2007) Identification of a mechanism of photoprotective energy dissipation in higher plants. *Nature* **450,** 575–578.

Ruban A.V., Johnson M.P. & Duffy C.D.P. (2012) The photoprotective molecular switch in the photosystem II antenna. *Biochimica et Biophysica Acta* **1817,** 167–181.

Sabater B. & Martín M. (2013) Chloroplast control of leaf senescence. In *Plastid Development in Leaves During Growth and Senescence, Advances in Photosynthesis and Respiration* (eds B. Biswal, K. Krupinska & U.C. Biswal), pp. 529–550. Springer, Dordrecht.

Schurr U., Walter A. & Rascher U. (2006) Functional dynamics of plant growth and photosynthesis – from steady-state to dynamics – from homogeneity to heterogeneity. *Plant, Cell and Environment* **29,** 340–352.

Siffel P., Santrucek J., Lang M., Braunová Z., Simkova M., Synkova H. & Lichtenthaler H.K. (1993) Age dependence of photosynthetic activity, chlorophyll fluorescence parameters and chloroplast ultrastructure in aurea and green forms of *Nicotiana tabacum* Su/su mutant. *Photosynthetica* **29,** 81–94.

Šesták Z. & Čatský J. (1962) Intensity of photosynthesis and chlorophyll content as related to leaf age in (*Nicotiana sanderae* hort. *Biologia Plantarum* **4,** 131–140.

Takahashi S. & Badger M.R. (2011) Photoprotection in plants: a new light on photosystem II damage. *Trends in Plant Science* **16,** 53–60.

Thiele A., Winter K. & Krause G.H.G. (1997) Low inactivation of D1 protein of photosystem II in young canopy leaves of *Anacardium excelsum* under high-light stress. *Journal of Plant Physiology* **151,** 286–292.

Thordal-Christensen H., Zhang Z., Wei Y. & Collinge D.B. (1997) Subcellular localization of H_2O_2 accumulation in papillae and hypersensitive response

during the barley-powdery mildew interaction. *The Plant Journal* **11,** 1187–1194.

Tichá I. (1985) Ontogeny of leaf morphology. In *Photosynthesis During Leaf Development* (ed. Z. Sestak), pp. 16–50. Academia, Prague.

Tsukaya H. (2013) Leaf development. *The Arabidopsis Book/American Society of Plant Biologists* **11,** e0163.

Umena Y., Kawakami K., Shen J.-R. & Kamiya N. (2011) Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. *Nature* **473,** 55–60.

van Amerongen H. & Croce R. (2013) Light harvesting in photosystem II. *Photosynthesis Research* **116,** 251–263.

van Lijsebettens M. & Clarke J. (1998) Leaf Development in Arabidopsis. *Plant Physiology and Biochemistry* **36,** 47–60.

Walter A., Rascher U. & Osmond B. (2004) Transitions in photosynthetic parameters of midvein and interveinal regions of leaves and their importance during leaf growth and development. *Plant Biology* **6,** 184–191.

Ware M.A., Belgio E. & Ruban A.V. (2015) Comparison of the protective effectiveness of NPQ in Arabidopsis plants deficient in PsbS protein and zeaxanthin. *Journal of Experimental Botany* **66,** 1259–1270.

Wientjes E., van Amerongen H. & Croce R. (2013) Quantum yield of charge separation in photosystem II: functional effect of changes in the antenna size upon light acclimation. *The Journal of Physical Chemistry B* **117,** 11200–11208.

Woo H.R., Chung K.M., Park J.H., Oh S.A., Ahn T., Hong S.H., . . . Nam H.G. (2001) ORE9, an F-Box protein that regulates leaf senescence in Arabidopsis. *The Plant Cell* **13,** 1779–1790.

Wraight C.A. & Crofts A.R. (1970) Energy-dependent quenching of chlorophyll *a* fluorescence in isolated chloroplasts. *European Journal of Biochemistry* **17,** 319–327.

Zeng X.-Q., Chow W.S., Su L.-J., Peng X.-X. & Peng C.-L. (2010) Protective effect of supplemental anthocyanins on Arabidopsis leaves under high light. *Physiologia Plantarum* **138,** 215–225.

Zhang J., Chen C., Zhang D., Li H., Li P. & Ma F. (2014) Reactive oxygen species produced via plasma membrane NADPH oxidase regulate anthocyanin synthesis in apple peel. *Planta* **240,** 1023–1035.

Zimmermann P., Heinlein C., Orendi G. & Zentgraf U. (2006) Senescence-specific regulation of catalases in *Arabidopsis thaliana* (L.) Heynh. *Plant, Cell and Environment* **29,** 1049–1060.

Received 9 February 2015; accepted for publication 14 May 2015

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Photosynthetic dynamics of *Arabidopsis thaliana* plants aged 3 weeks. (a) Relationship between photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters. The continuous line represents the theoretical yield, calculated by Eqn 1 of Materials and Methods. Error bars show the SEM ($n = 30$). (b) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (c) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/(cb + xb)$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

Figure S2. Photosynthetic dynamics of *Arabidopsis thaliana* plants aged 5 weeks. (a) Relationship between photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters. The continuous line represents the theoretical yield, calculated by equation 1 of materials and methods. Error bars show the SEM ($n = 30$). (b) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (c) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

Figure S3. Photosynthetic dynamics of *Arabidopsis thaliana* plants aged 9 weeks. (a) Relationship between photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters. The continuous line represents the theoretical yield, calculated by Eqn 1 of Materials and Methods. Error bars show the SEM ($n = 30$). (b) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (c) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

Figure S4. Photosynthetic dynamics of *Arabidopsis thaliana* plants aged 10 weeks. (a) Relationship between photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters. The continuous line represents the theoretical yield, calculated by Eqn 1 of Materials and Methods. Error bars show the SEM ($n = 30$). (b) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (c) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

Figure S5. Photosynthetic dynamics of *Arabidopsis thaliana* plants aged 12 weeks. (a) Relationship between photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters. The continuous line represents the theoretical yield, calculated by Eqn 1 of Materials and Methods. Error bars show the SEM ($n = 30$). (b) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (c) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

Figure S6. Anthocyanin visible absorption spectra profile measured in leaves of 7-week-old *Arabidopsis thaliana* plants. The absorbance was measured with a spectrophotometer (U-330, Hitachi, Tokyo, Japan) using wavelengths ranging from 400 to 750 nm, with 1 nm of resolution. The spectra represent the average of extracts from three independent leaves.

Figure S7. Electron transport rate versus light intensity curves measured in *Arabidopsis thaliana* plants from 1 to 13 weeks old. Lines represent regression fit curves (Exponential Rise to Maximum; Single, 2 Parameter $f = a^*(1 - \exp(-b^*x))$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

Figure S8. Photosynthetic dynamics of intermediate leaves in *Arabidopsis thaliana* aged 8 weeks. (a) Relationship between

photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters. The continuous line represents the theoretical yield, calculated by equation 1 of materials and methods. Error bars show the SEM ($n = 30$). (b) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (c) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

Figure S9. Photosynthetic dynamics of inner leaves in *Arabidopsis thaliana* aged 8 weeks. (a) Relationship between photosystem II (PSII) yield (closed circles), qPd (open circles) and non-photochemical fluorescence quenching (NPQ) parameters. The continuous line represents the theoretical yield, calculated by Eqn 1 of Materials and Methods. Error bars show the SEM ($n = 30$). (b) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (c) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; $f = axb/cb + xb$) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc.).

Table S1. Physiological markers of the four ontogenetic phases studied (juvenile, adult, reproductive and senescent). *Arabidopsis thaliana* plants were grown at 24 °C (day)/18 °C (night), 45% humidity, 175 ± 20 μmol m^{-2} s^{-1} actinic light and photoperiod of 10 h.

Table S1. Physiological markers of the four ontogenetic phases studied (juvenile, adult, reproductive and senescent). *Arabidopsis thaliana* plants were grown at 24°C (day)/18°C (night), 45% humidity, 175± 20 µmol m^{-2} s^{-1} actinic light and photoperiod of 10 hours.

Plant phase	Age (weeks)	Rosette size	Inflorescence features	Most external leaf physiological markers	Most external leaf area density (mg cm^{-2})*
Juvenile	0-3	0-6 rosette leaves	Not present	Rounded, reduced or absent epinastic curvature	23.02±1.36c (2 weeks)
Adult	4-6	20-100% rosette diameter	Not present	Oblong, serrated border, presence of epinastic curvature	33.23±2.00a (6 weeks)
Reproductive	7-8	100% rosette diameter	Vertical flowering branch emerged	Oblong, serrated border, increase of epinastic curvature, scarce chlorosis spots	35.53±3.32a (8 weeks)
Senescent	>8	100% rosette diameter	Gradual increment in the number of matured siliques	Oblong, serrated border, high epinastic curvature, large and abundant chlorosis spots	27.87±1.88b (11 weeks)

*Average and standard deviation (n=6). Different letters mean significant differences tested by Tukey's test, with $p \leq 0.05$.

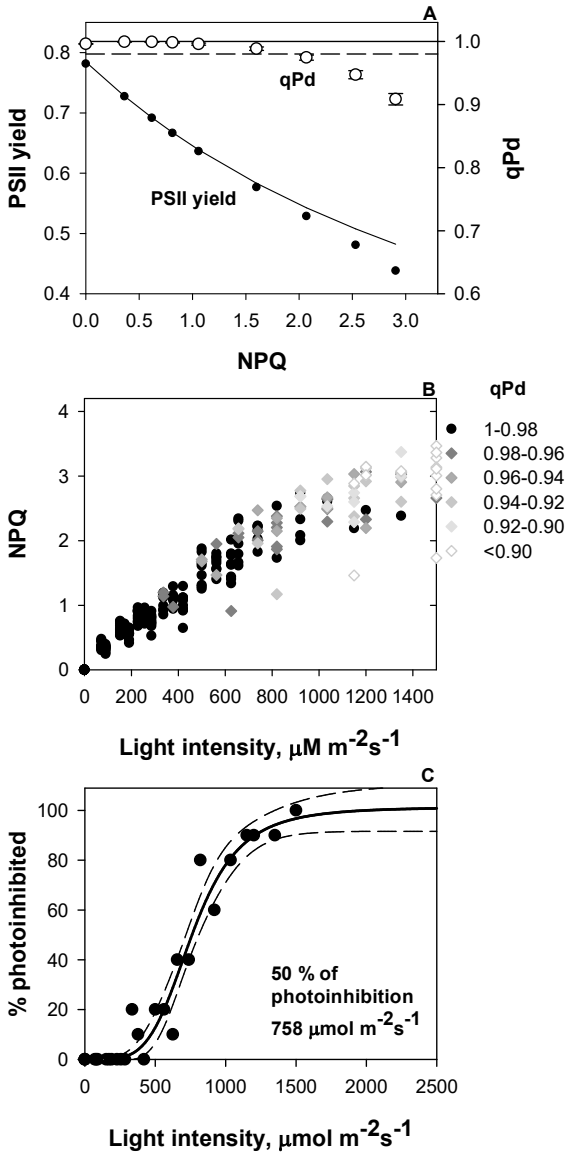

Fig. S1. Photosynthetic dynamics of *Arabidopsis thaliana* plants aged 3 weeks. (A) Relationship between PSII yield (closed circles), qPd (open circles) and NPQ parameters. The continuous line represents the theoretical yield, calculated by equation 1 of materials and methods. Error bars show the SEM (n=30). (B) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (C) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; f=axb/cb+xb) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc., Chicago, IL, USA).

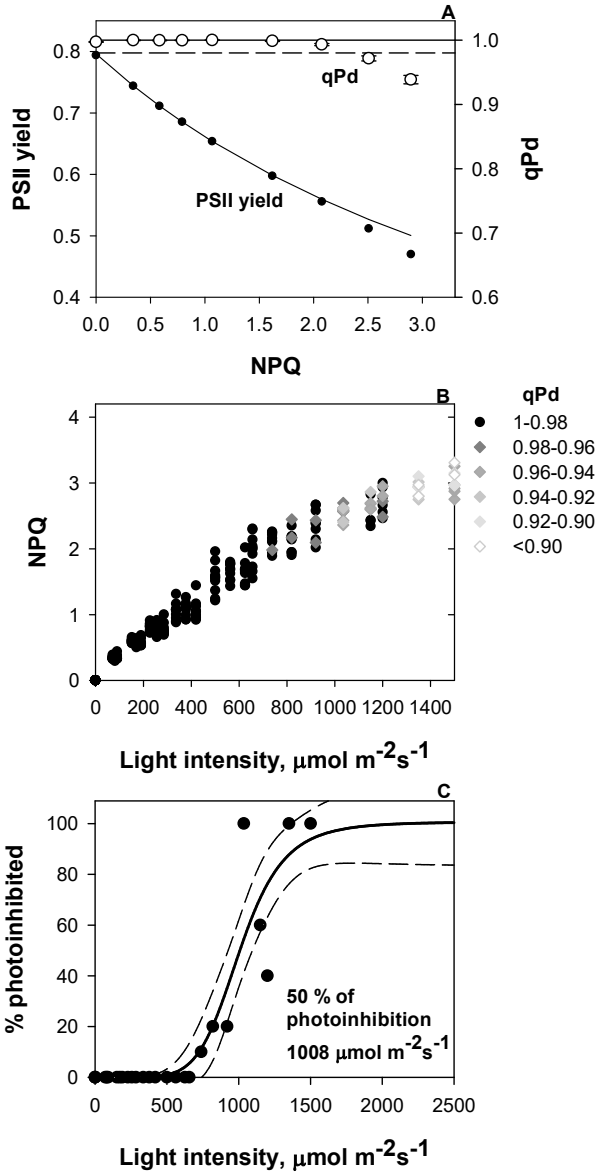

Fig. S2. Photosynthetic dynamics of *Arabidopsis thaliana* plants aged 5 weeks. (A) Relationship between PSII yield (closed circles), qPd (open circles) and NPQ parameters. The continuous line represents the theoretical yield, calculated by equation 1 of materials and methods. Error bars show the SEM (n=30). (B) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (C) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; f=axb/cb+xb) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc., Chicago, IL, USA).

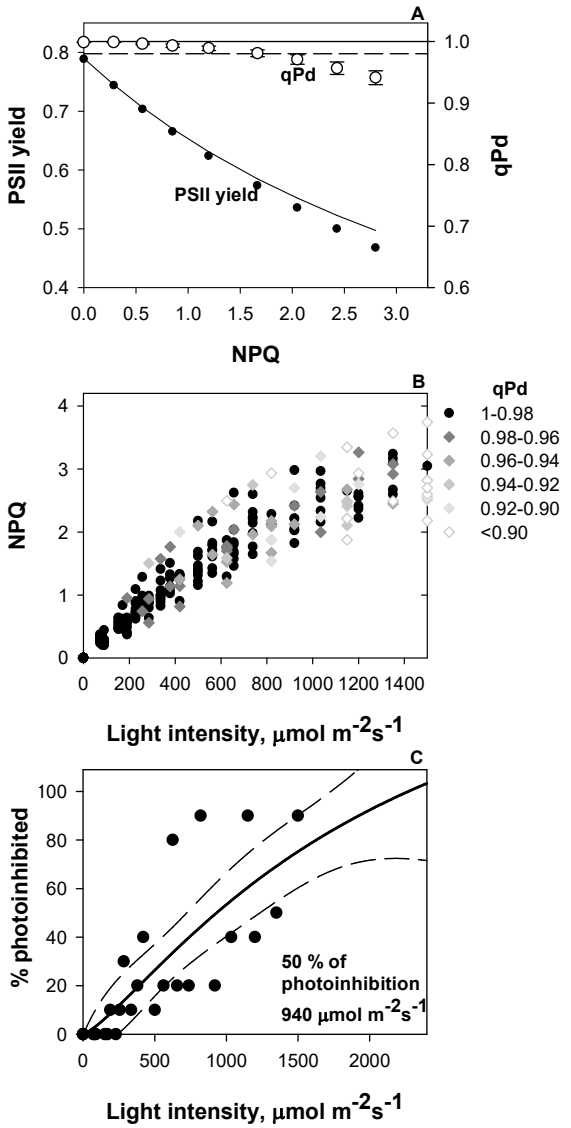

Fig. S3. Photosynthetic dynamics of *Arabidopsis thaliana* plants aged 9 weeks. (A) Relationship between PSII yield (closed circles), qPd (open circles) and NPQ parameters. The continuous line represents the theoretical yield, calculated by equation 1 of materials and methods. Error bars show the SEM (n=30). (B) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (C) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; f=axb/cb+xb) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc., Chicago, IL, USA).

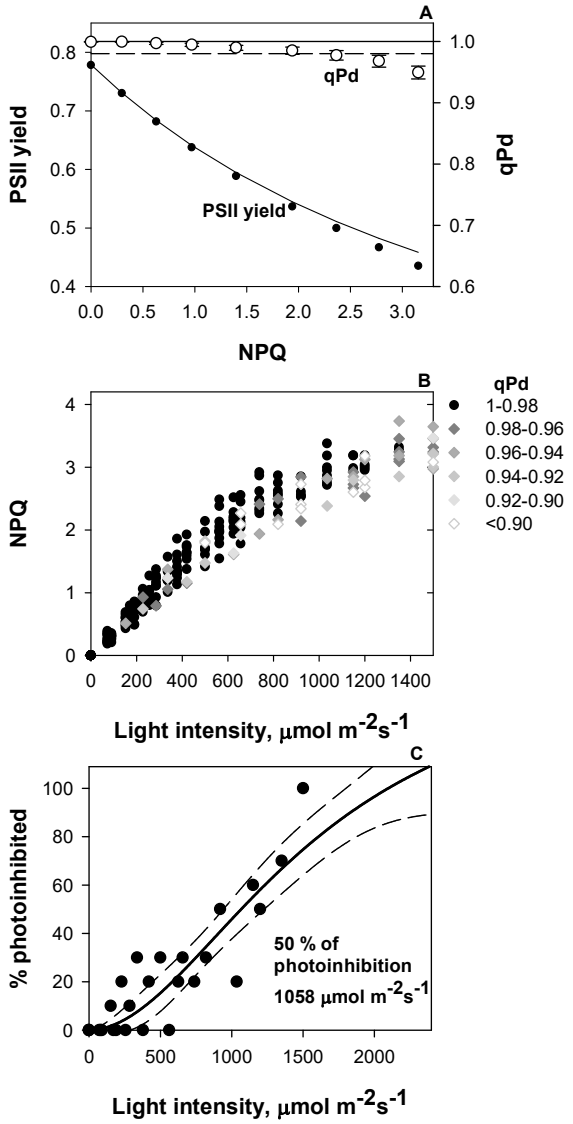

Fig. S4. Photosynthetic dynamics of *Arabidopsis thaliana* plants aged 10 weeks. (A) Relationship between PSII yield (closed circles), qPd (open circles) and NPQ parameters. The continuous line represents the theoretical yield, calculated by equation 1 of materials and methods. Error bars show the SEM (n=30). (B) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (C) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; f=axb/cb+xb) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc., Chicago, IL, USA).

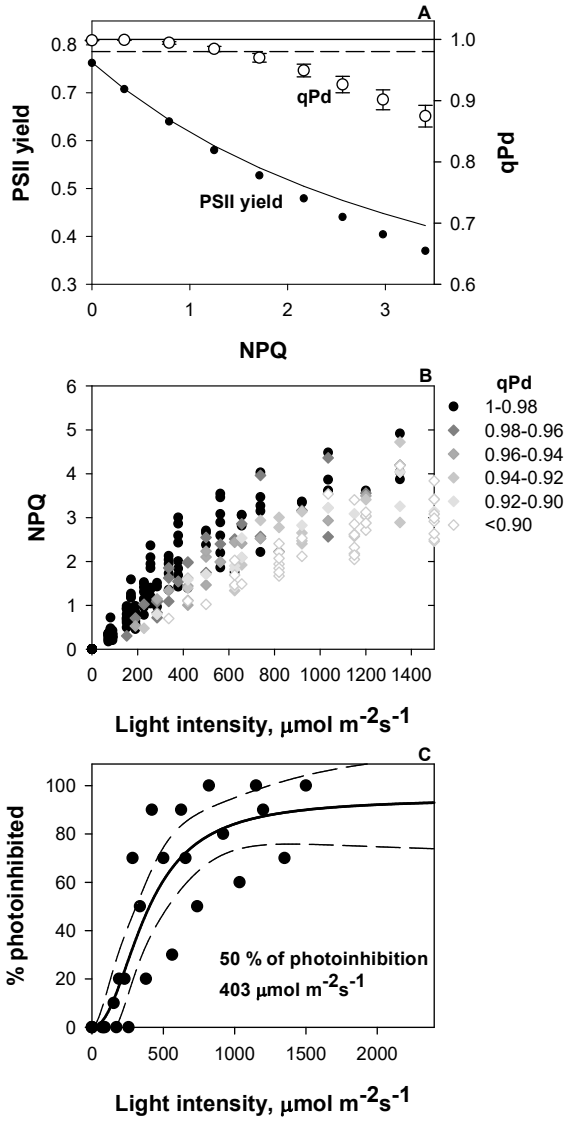

Fig. S5. Photosynthetic dynamics of *Arabidopsis thaliana* plants aged 12 weeks. (A) Relationship between PSII yield (closed circles), qPd (open circles) and NPQ parameters. The continuous line represents the theoretical yield, calculated by equation 1 of materials and methods. Error bars show the SEM (n=30). (B) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (C) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; f=axb/cb+xb) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc., Chicago, IL, USA).

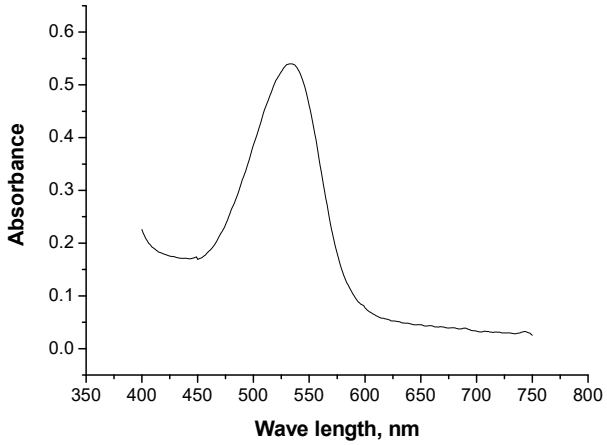

Fig. S6. Anthocyanin visible absorption spectra profile measured in leaves of 7 week old *Arabidopsis thaliana* plants. The absorbance was measured with a spectrophotometer (U-330, Hitachi, Tokyo, Japan) using wavelengths ranging from 400 to 750 nm, with 1 nm of resolution. The spectra represent the average of extracts from 3 independent leaves.

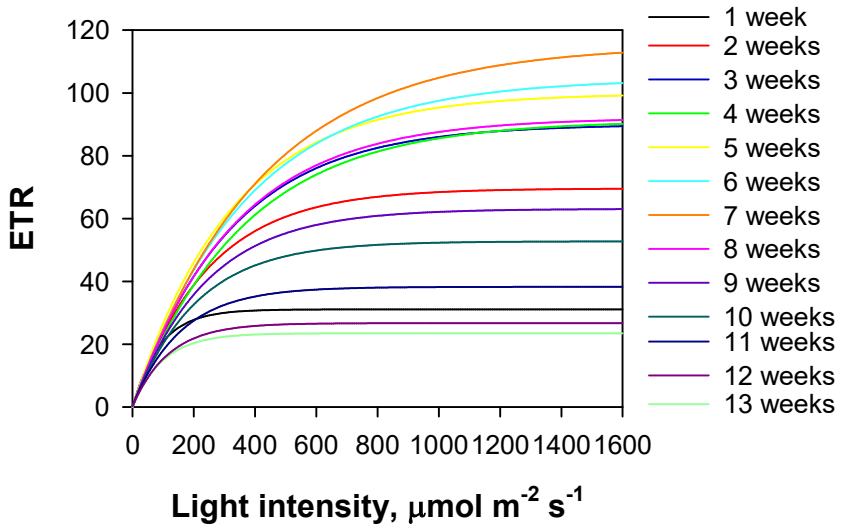

Fig. S7. Electron transport rate vs light intensity curves measured in *Arabidopsis thaliana* plants from 1 to 13 weeks old. Lines represent regression fit curves (Exponential Rise to Maximum; Single, 2 Parameter f = a*(1-exp(-b*x))) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc., Chicago, IL, USA).

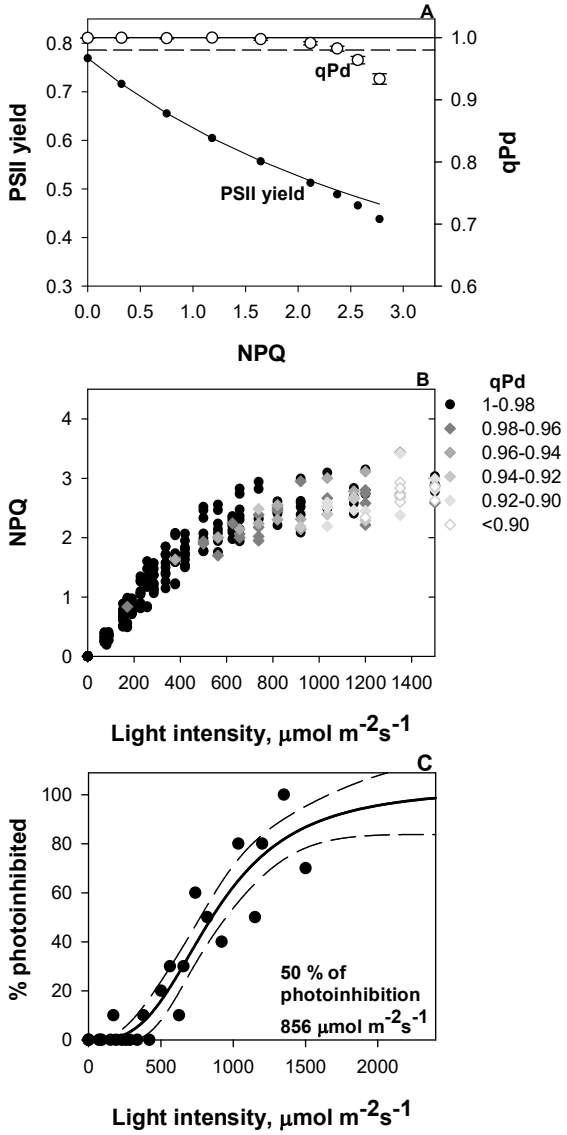

Fig. S8. Photosynthetic dynamics of intermediate leaves in *Arabidopsis thaliana* aged 8 weeks. (A) Relationship between PSII yield (closed circles), qPd (open circles) and NPQ parameters. The continuous line represents the theoretical yield, calculated by equation 1 of materials and methods. Error bars show the SEM (n=30). (B) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (C) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; f=axb/cb+xb) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc., Chicago, IL, USA).

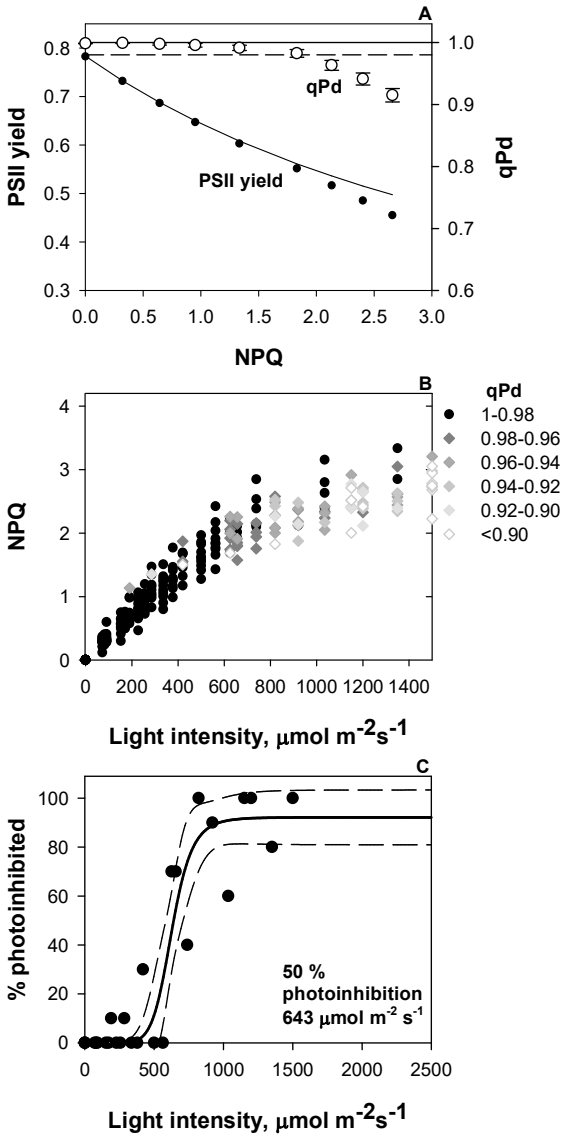

Fig. S9. Photosynthetic dynamics of inner leaves in *Arabidopsis thaliana* aged 8 weeks. (A) Relationship between PSII yield (closed circles), qPd (open circles) and NPQ parameters. The continuous line represents the theoretical yield, calculated by equation 1 of materials and methods. Error bars show the SEM (n=30). (B) Relationship between NPQ, light intensity and qPd parameters. qPd is represented by diamond symbols, with the grey-scale shading reflecting the extent of photodamage. (C) Percentage of photoinhibited leaves. Lines represent regression fit curves (sigmoidal, Hill, three parameter; f=axb/cb+xb) with 95% confidence values plotted using SigmaPlot12 (Systat Software, Inc., Chicago, IL, USA).

INTERCHAPTER

In the chapter II, it was investigated the use of new fluorescence-based methodology that uses the coefficient of photochemical quenching measured in the dark following illumination (qPd) to assess the intactness of RCIIs. This methodology was tested in a practical physiological problem regarding light tolerance changes during *Arabidopsis thaliana* ontogenesis. The data obtained highlighted the great potential of this technique for the tracking of the exact onset of photoinhibition in illuminated plants. Differently from other methodologies related to photoinhibition, which demands the light exposure interruption (Fv/Fm) or tissue maceration (D1 blotting), qPd, is a fast, non-destructive and efficient method to quantify photoinhibition. Moreover, the relationship between qPd and NPQ provides a new perspective of NPQ study, which encompass the calculation of the exact fraction of NPQ formed that confers photoprotection to RC, this parameter was called pNPQ. Light tolerance in *A. thaliana* plants during ontogenesis exhibited a close relation with efficiency in pNPQ generation. In addition, pNPQ formation efficiency was associated with higher amounts of chlorophyll molecules and reduced levels of reactive oxygen species (ROS). However, as the relationship between pNPQ, classical NPQ components such as qE and plant physiological problems still not completely clear, further studies regarding these mechanisms underlying NPQ were grouped in the next chapter.

CHAPTER III

(Unpublished data - Manuscript draft)

Original research

Mechanisms underlying phototolerance dynamics during Arabidopsis ontogenesis

Fabricio E. L. Carvalho[1,2], Alexander V. Ruban[2*], Joaquim A. G. Silveira[1]

[1]Department of Biochemistry and Molecular Biology, Federal University of Ceará, Fortaleza/CE – Brazil, Zip Code 60451-970.

[2]School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK

Corresponding author:

Professor Alexander V. Ruban

e-mail: a.ruban@qmul.ac.uk

tel.: +44(0)2078826314

fax : +44(0)2078827732

School of Biological and Chemical Sciences

Queen Mary University of London

Fogg Building, Mile End Road

London, E14NS

United Kingdom

Abstract - Plants are sessile organisms that frequently have to face oscillations in light availability. Because of daily or seasonal variations, plants are constantly exposed to light intensities that are lower than necessary for optimal metabolic activity or higher enough to cause damage to photosystem II (PSII) reaction centers (RCs) and photoinhibition. Therefore, plants evolved several strategies to cope with these adverse conditions. The protective non-photochemical quenching (pNPQ) is a recently described photochemical parameter, related to the specific amount of formed NPQ, which confers photoprotection to PSII RCs. Recently, was demonstrated that the pNPQ formation in *A. thaliana* is age-dependent and close related to the amount of light that plants could tolerate during different phases of ontogenesis. Thus, aiming to clarify the mechanisms underlying the age-dependent pNPQ formation, PsbS protein accumulation, ∆pH dynamics, carotenoid contents and ratios of PSII/PSI super-complexes in juvenile, mature and senescent *Arabidopsis* plants were assessed. Juvenile plants exhibited higher PsbS amounts compared to senescent plants and higher zeaxanthin accumulation in comparison to mature and senescent plants. However, the capability of ∆pH formation in mature and senescent plants was higher than that of juvenile plants. Despite a higher ∆pH formation observed in senescent plants, the combination of lower amounts of PsbS and zeaxanthin might have contributed to restriction of pNPQ formation during senescence. In juvenile plants, the lower ∆pH formation and PSII/PSI ratio probably were decisive to restrict the pNPQ formation. Nevertheless, the mature plants exhibited the most advantageous combination of NPQ underlying mechanisms, which probably contributed to the most efficient pNPQ formation and subsequently the higher light intensities tolerated. However, further studies are still needed regarding the molecular mechanisms underlying pNPQ.

Key-words: ontogenesis, light tolerance, photoinhibition, protective NPQ

Introduction

Photosynthesis is a complex metabolic process closely related to efficient sunlight capture and subsequently reduction of atmospheric CO_2 (Raven, 1984). During photosynthesis, the potential energy generated from proton/electrochemical gradient in thylakoid membranes is subsequently employed for ATP and NADPH production, which are used in the Calvin-Benson cycle as energy donor and reducing power, respectively, for CO_2 assimilation (Arnon et al., 1954; Hill and Bendall, 1960; Paul and Foyer, 2001). Light irradiance intensities change dramatically as consequence of the seasonal variation, the normal photoperiod or even an eventual shadowing day (Ruban, 2014). Because plants are sessile organisms, during evolution, natural selection pressure has driven plants to develop several strategies to cope with the light fluctuations (Ruban, 2014). These strategies are especially represented by mechanisms related to optimization of light capture during shadowing and excess energy drain during excessive light stress.

Under light, electrons are transported from PSII reaction centers (RCII) by electron/proton carriers in the thylakoid membrane (PETC). The generation of a redox- and proton energy gradient is a direct consequence of this process. However, as the events related to CO_2 assimilation in Calvin-Benson cycle are much slower than the charge separation event in PSII, the occurrence of excess electrons in PETC is very usual (Ruban, 2014). The excess energy could lead to reactive oxygen species (ROS) generation at both PSII and photosystem I sites (Asada, 2006; Zulfugarov et al., 2014). Excessive ROS could cause deleterious effects to lipid membranes, proteins and DNA, eventually leading to organism death (Apel and Hirt, 2004). Fortunately, the occurrence of both mechanisms of excess energy formation avoidance and ROS scavenging are widespread in higher plants (Takahashi and Badger, 2011). Among the mechanisms related to excess energy avoidance, the non-photochemical quenching (NPQ) is one of the most important (Ruban et al., 2012). NPQ is composed of four major processes:

state transition (qT), photoinhibition (qI), heat dissipation (qE) and zeaxanthin-dependent (qZ) quenching (Baker, 2008; Ruban, 2014).

The major and fastest component of NPQ, qE, acts in RCII photoprotection and is related to the excess energy dissipation as heat (Baker, 2008; Ruban et al., 2012). The site of qE formation is believed to be the light harvesting antenna of PSII, but this issue is still under discussion (Horton et al., 1996; Ruban et al., 2007; Ahn et al., 2008). In contrast, a relative consensus regarding the mechanisms underlying qE formation has been established in the last decades. Firstly, the increase in the lumen pH is the key event for triggering qE (Krause and Behrend, 1986; Ruban et al., 1992). Secondly, the roles of zeaxanthin and PsbS protein in this event are to increase the sensitivity of the membrane to pH changes by binding to antenna proteins (Demmig-Adams and Adams, 1992; Funk et al., 1995; Li et al., 2002; Niyogi et al., 2005). After zeaxanthin and PsbS binding, a new state of aggregation at light harvesting complexes is achieved, allowing excess energy dissipation as heat (Ruban et al., 1992; Farber et al., 1997; Ruban et al., 1999; Betterle et al., 2009; Johnson and Ruban, 2011).

During plant ontogenesis, several physiological changes take place (Avery and Avery Jr, 1933; Milthorpe, 1959; Hopkinson, 1964). In *Arabidopsis thaliana*, the emergence of the radicle, hypocotyl and cotyledon occur around 4-5 days after sowing, followed by a succession of distinct phases regarding to shoot growth (Boyes et al., 2001). The juvenile phase consists of vegetative growth, when the leaves are smaller and rounded, with trichomes on the adaxial side and a lack of serrations along the margin (Kerstetter and Poethig, 1998; Kozuka et al., 2005). The adult phase is characterized by a bigger rosette, in terms of number of leaves and total diameter (Boyes et al., 2001). The leaves are larger, elongated, the trichomes are present on both adaxial and abaxial surfaces, and the serrations along the margin are frequent (Kerstetter and Poethig, 1998; Tsukaya, 2002). The reproductive phase is marked by inflorescence and is strongly regulated by the photoperiod prehistory (Amasino, 2010). The last phase (senescence) marks the end of the plant life cycle. This phase is recognized by the gradual

appearance of necrosis on the leaves, followed by abscission and plant death (Matile et al., 1992).

Recently, Carvalho et al. (2015) studying the roles of NPQ in photoprotection of Arabidopsis plants during ontogenesis, employed a new methodology (Ruban and Murchie, 2012) for photoinhibition detection. This methodology is based on measurement of photochemical quenching in the dark (qPd) and subsequent estimation of the NPQ fraction effectively associated with photoprotection, the protective NPQ (pNPQ). Arabidopsis plants exhibited higher tolerance to light during the early reproductive phase. This tolerance was closely related with total chlorophyll content and pNPQ formation (Carvalho et al., 2015). However, an important question remains unknown: which biochemical changes, related to ontogenesis, could be underlying the different pNPQ throughout Arabidopsis life cycle? To answer this question, it is noteworthy that the main photoprotective NPQ component, qE, is dependent on three main mechanisms: a) ∆pH gradient accumulation between the lumen and the stromal sides of the thylakoid membranes, b) signal transduction from this ∆pH gradient to the zeaxanthin deepoxidase activity promoted by PsbS protein and c) the zeaxanthin binding to the antenna complexes. Thus, we hypothesized that plants at different ontogenetic phases might exhibit consistent modulation of these three above mechanisms.

Aiming to test our hypothesis, *A. thaliana* plants (Col-0) at three distinct phases of ontogeny, juvenile (3 weeks old), reproductive (8 weeks old) and senescent (12 weeks old) were evaluated in regarding to the PsbS protein content, ∆pH accumulation dynamics, carotenoid contents and ratios of PSII/PSI super-complexes. Taken together, our data evidenced that both juvenile and senescent plants were restricted in important mechanisms related to NPQ formation. Thus, it is reasonable to suggest that differences in pNPQ formation are dependent on the balance between PsbS content, ∆pH accumulation and violaxanthin/zeaxanthin de-epoxidase activity, according to our hypothesis. Nevertheless, the mature plants exhibited the most advantageous combination of NPQ underlying mechanisms. This feature exhibited by

reproductive plants was probably detrimental to the most efficient pNPQ formation and subsequently higher light intensity tolerance.

Material and methods

Plant growth

Arabidopsis thaliana plants, ecotype Col-0, were sown into 0.3 L pots containing a 6:6:1 mixture of soil, potting compost (John Innes Manufacturers Association, Berkshire, UK) and perlite. The germination occurred in a growth chamber with 24°C (day)/18°C (night), 45% humidity, 100 μmol m^{-2} s^{-1} actinic light intensity, and 10 h photoperiod. After 1 week the plants were transferred to growth shelves with the same previously described conditions, but under 175 μmol m^{-2} s^{-1} actinic light. Plants were grown under these conditions for up to 12 weeks. For ΔpH fluorimetric determination, 30 leaves from independent plants were used. In every measurement only the leaves most externally positioned in the rosette, at respective ontogenetic phases, were utilized. The same criteria were adopted for collecting the leaves used for PsbS, carotenoids and ratios of photosynthetic super-complexes determinations.

Chloroplasts and thylakoids membranes preparation

Intact chloroplasts were prepared by homogenizing fresh tissue (5-10 g) in ice-cold grinding medium (330 mM sorbitol, 5 mM $MgCl_2$, 10 mM Na_4P_2O7, pH 6.5, 2 mM iso-ascorbate) with a polytron. The homogenate was then filtered through four layers of muslin followed by two layers of muslin and one layer of cotton wool. The filtrate was centrifuged for 10 min at 5,000 g, and the chloroplast-enriched pellet was resuspended in wash buffer (330 mM

sorbitol, 10 mM MES, pH 6.5). In order to isolate stacked thylakoid membranes, chloroplast-enriched preparation was further centrifuged by 10-min at 5,000 g. The pellet was then resuspended in 5 mM $MgCl_2$ for 30 s to lyse any remaining intact chloroplasts, followed by an equal volume of osmoticum (660 mM sorbitol, 20 mM KCl, 2 mM EDTA, and 100 mM HEPES, pH 6.5). After further centrifugation, thylakoids were resuspended in 20 mM Bis-Tris (pH 6.5), 5 mM $MgCl_2$.

Determination of ∆pH gradient in isolated chloroplasts

The ∆pH was determined by measuring 9-aminoacridine (9-AA) fluorescence using the dual-ENADPH and Dual-DNADPH modules for the Dual- PAM-100 chlorophyll fluorescence analyser (Walz, Germany). A total volume of 1.4 mL of intact chloroplasts suspension was measured in a quartz cuvette at a 0.018 mg mL^{-1} chlorophyll concentration, under continuous stirring and in the presence of 0.001 mM 9-AA. The reaction medium contained 450 mM sorbitol, 20 mM HEPES, 20 mM MES, 20 mM sodium citrate, pH 8.0, 10 mM EDTA, 10 mM $NaHCO_3$, 0.1 % BSA, 5 mM $MgCl_2$. Ascorbate was omitted from all buffers to prevent further de-epoxidation taking place during illumination. Where mentioned, 0.4 mM DAD (diaminodurene) or 0.1 mM MV (methyl viologen), both in their reduced form, was added to intact chloroplasts to stimulate LEF. Excitation was provided by 365 nm LEDs and fluorescence emission was detected between 420 and 580 nm. ∆pH was estimated from the values of 9-AA quenching, using the equation: $\Delta pH = \log[1/(1 - Q) + Q/(1 - Q)](Vout/Vin)$, where Q is the level of 9-AA quenching, Vout is the sample volume (1.4 mL), and Vin is the lumen volume (0.056 mL mg^{-1} of chlorophyll) (Schuldiner et al., 1972).

PsbS western blotting

Leaf extracts (1.2 µg chlorophyll) were first separated by SDS-PAGE (Laemmli, 1970). Subsequently, they were electrophoretically transferred to a nitrocellulose membrane (Towbin et al., 1979). Next, the membrane was blocked overnight with 5% (m/v) non-fat milk in 100 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl, and 0.05% Tween-20 and incubated with the primary polyclonal antibodies against PsbS (Agrisera©, Sweden). The specific polypeptide was revealed after incubation with the secondary antibodies conjugated with peroxidase (Agrisera©, Sweden). ECL plus Western Blotting Detection System® was used for detection (GE Healthcare Life Sciences©, UK), according to manufacturer's instructions.

Carotenoid and chlorophyll contents

Extraction of total carotenoids and chlorophylls from ~100 mg Arabidopsis fresh leaves was carried out by maceration in the presence of 1 mL ice cold 80% (v/v) acetone in 2.5 mM sodium phosphate buffer, pH 7.8, as previously described (Porra et al., 1989). The leaves were previously illuminated – 380 µmol m^{-2} s^{-1}, for 45 min, in the presence of saturating N_2. Separation and quantification of these pigments were achieved with a LiChrospher 100 RP-18 column with 5-bm particle size (Merck©, Germany) coupled to a HPLC system (Thermo Scientific™/Dionex™, USA). The following solvent solutions were utilized: solvent A (87 % acetonitrile, 10 % methanol and 3 % 100 mM tris-HCl buffer, pH 8.0) and solvent B (80 % methanol, 20 % Hexane). The run profile consisted of: 0 min to 9 min, 100% A; 9.1 min to 12.5 min, 0% to 100% B; 12.6 min to 18 min, 100% B; 18.1 min to 19 min, 100% to 0% B and 19.1 min to 23 min, 100% A. The flow was set to 1 mL min^{-1}. Eluted pigments were monitored at 440 nm. Calculation of the pigment concentrations was done on the base of pure pigment standard concentrations. Neoxanthin and violaxanthin were previously isolated by thin-layer chromatography. Chl *a* and *b* were purchased from Serva© (Germany), lutein and β-carotenoids from Sigma© (USA) and zeaxanthin was a kind gift from Hoffmann-LaRoche (Basel,

Switzerland). The factors used were respectively: 2772 (neoxanthin), 3211 (violaxanthin), 2192 (lutein), 2707 (Zea), 1609 (Chl a), 1298 (Chl b), and 2001 (β-carotenoids), expressed as peak area ($mm^2.\rho mol^{-1}$) of the respective pigment (Farber et al., 1997).

Separation of the Photosynthetic protein supercomplexes

Gel filtration (fast protein liquid chromatography, FPLC) was performed with stacked thylakoid membranes obtained as described previously (Ruban et al., 2006). Firstly, 1.0 mg chlorophyll mL^{-1} of isolated thylakoids were solubilised with n-dodecyl-α-D-maltoside to 1% final concentration and incubated for 1 min. After centrifugation at 16000 x *g* for 1 min, 25 °C, the supernatant was filtered through a 0.45 μm filter and loaded on a Superdex 200 HR 10/30 exclusion chromatography column® (Amersham-Pharmacia©, UK) coupled to an Amersham-Pharmacia Acta purifier system®. The mobile phase utilized consisted of 20 mM BisTris (pH 6.5), 5 mM $MgCl_2$ and 0.03% α-DM and the flow rate was set as 0.5 ml/min. The 670nm, 700nm and 260nm absorbencies were monitored.

77 K fluorescence emission spectra

Stacked thylakoid membranes were obtained as previously described by Ruban et al. (2006). The thylakoid membranes isolated were subsequently injected into the sample holder and shock-frozen in liquid N_2 (Ware et al., 2015). Measurements were performed at 77 K using a custom-made cryostat and Jobin Yvon FluoroMax-3 spectrophotometer. Fluorescence spectral resolution was set as 1 nm, with 5 nm of spectral bandwidth and excitation defined as 435 nm.

Statistical design

The experiment was arranged in a completely randomized design at three plant ages (juvenile, reproductive and senescent) with four independents replicates. Each replicate consisted of an individual pot containing one independent plant. Data were analysed by ANOVA, and the means were compared using the Tukey's test at 0.05 confidence level using the SigmaPlot® 12 software (Systat Software, Inc., USA).

Results

Arabidopsis reproductive phase plants exhibited the most advantageous combination of NPQ formation underlying mechanisms

Our hypothesis is that age-dependent pNPQ formation differences previously reported for *A. thaliana* plants are a consequence of deep changes of the mechanisms underlying the NPQ formation. Aiming to test this hypothesis, *A. thaliana* plants at three different phases of ontogeny were studied in terms of PsbS protein amount, capability of ∆pH formation and zeaxanthin/violaxanthin balance.

Plants at juvenile phase were collected 3 weeks after germination. They were characterized by the presence of approximately 6 leaves in the rosette (less than 20% of rosette final diameter), rounded leaves with scarce trichomes and reduced or absent epinastic curvature (Fig. 1A). The mature phase plants were collected at 8 weeks after germination. They were characterized by the presence of emerging first reproductive branches, bigger rosettes than those at juvenile phase (100% of final rosette diameter), and several leaves per rosette, which are oblong, with serrated border and increased epinastic curvature. Moreover, the first scarce leaf chlorosis spots were seen at this phase (Fig. 1B). Finally, the senescent phase plants were collected 12 weeks' post-germination. These plants presented complete reproductive

maturation, and exhibited several mature siliques in the branches. The leaves were oblong with serrated border, presented higher epinastic curvature in comparison to those at the mature phase and large and abundant chlorosis spots in the leaves (Fig. 1C).

The PsbS protein amount, which is associated with the binding of zeaxanthin to antenna complexes and subsequent formation of heat component of NPQ (qE), was evaluated by western blotting. Plants at the juvenile phase presented approximately 60% higher PsbS amount in comparison to senescent plants (Fig. 2; Tukey's test, $p \leq 0.05$). In contrast, mature plants presented no significant differences between juvenile or senescent plants (Fig. 2; Tukey's test, $p \leq 0.05$). The proton gradient formation (ΔpH) is a crucial event in the activation of de-epoxidation of violaxanthin to zeaxanthin and subsequently NPQ formation. In the current study, juvenile plants presented approximately 20 % and 30 % lower capability of ΔpH formation during exposure to light in comparison to mature and senescent plants, respectively (Fig. 3; Tukey's test, $p \leq 0.05$). In parallel, senescent plants presented around 15 % higher ability of ΔpH formation after light exposure in comparison to mature plants (Fig. 3; Tukey's test, $p \leq 0.05$). The zeaxanthin/violaxanthin balance is also a very important mechanism associated with qE formation. The conversion of violaxanthin to zeaxanthin, which occurs in response to light, is a vital event to NPQ formation, since zeaxanthin binding to LHCII leads to changes in this antenna-complex conformation, favouring the energy dissipation as heat (qE).

The juvenile plants presented higher de-epoxidation state (66 %) and higher zeaxanthin and anteroxanthin contents (144 % and 66 %, respectively), in comparison to senescent plants (Fig. 4 Tukey's test, $p \leq 0.05$). However, mature plants presented the higher total xanthophyll's cycle pool in comparison to juvenile (100 % higher) and senescent (270 % higher) plants (Fig.4 Tukey's test, $p \leq 0.05$). In parallel, mature phase plants also exhibited the highest content of neoxanthin (64 % and 32 %, in comparison to juvenile and senescent plants, respectively) and the lowest content of β-carotenoids, approximately 15 % of the values found for the other

studied ontogenetic phases (Fig.4 Tukey's test, $p \leq 0.05$). No significant differences of lutein content were detected among the ontogenetic phases studied (Fig.4 Tukey's test, $p \leq 0.05$).

Age-dependent differences in Photosynthetic protein super-complex ratios and PSII/PSI ratios

As PSII antenna super-complexes are the most likely site of q(E) formation (photoprotective heat-component of NPQ), the dynamics of thylakoidal photosynthetic super-complex content was investigated (Fig. 5). Plants at mature phase exhibited the highest content of PSII-LHCII super-complexes, PSI, PSII Core and LHCII trimmers in comparison to juvenile and mature plants (Table 1; Tukey's test, $p \leq 0.05$). In turn, plants at juvenile phase presented higher content of PSII-LHCII super-complexes, PSI, PSII Core and LHCII trimmers than senescent plants (Table 1; Tukey's test, $p \leq 0.05$). In opposition, the PSII/PSI ratio was higher in senescent plants in comparison to both the other ontogenetic phases, especially compared to juvenile phase, which represented about 30% of the senescence plant ratios. This relationship was also corroborated by 77 K fluorescence measurements, which also indicated a higher PSII/PSI ratio in senescent plants compared to the other two studied ontogenetic phases (Fig. 6; Table 2; Tukey's test, $p \leq 0.05$).

Discussion

In order to increase the knowledge of plant ontogenesis-dependent light tolerance, our research group has recently employed a novel methodology to advance our understanding of the dynamics of photoinhibition and photoprotection. These consisted of two new parameters: the dark photochemical quenching (qPd) determination, a new photosynthetic parameter close related to the onset of photoinhibition during light exposure (Ruban and Belgio, 2014; Ware et al., 2014; Giovagnetti and Ruban, 2015; Ware et al., 2015); and the protective non-

photochemical quenching (pNPQ), the specific amount of NPQ formed which is able to concretely lead to photoprotection (Ruban and Murchie, 2012; Ruban and Belgio, 2014; Ware et al., 2014; Giovagnetti and Ruban, 2015; Ware et al., 2015). Both these new fluorimetric tools to assess the dynamics of the photosynthetic response to light fluctuations during ontogenesis had previously been employed in *A. thaliana* studies done by our group (Carvalho et al., 2015).

A. thaliana plants display an efficient dynamic of phototolerance during ontogenesis (Nath et al., 2013; Carvalho et al., 2015). The light intensity tolerated increased from the first week after sowing until 8 weeks - from juvenile to mature phases, followed by a decrease until 13 weeks - from mature to senescent phases (Carvalho et al., 2015). This trend was correlated with pNPQ formation, indicating that this parameter could turn out to be a crucial factor in define the ontogenetic tolerance of light exposure in *A. thaliana* plants. Since utilization of pNPQ parameter as an indicative of the correlation of NPQ fraction with PSII photoprotection is very recent in the literature (Ruban and Murchie, 2012), its physiological relevance was not elucidated yet. However, previous studies on *Arabidopsis -npq4* (lacking PsbS protein), *-L17* (an overexpresser of PsbS protein), and *-npq1* (unable to convert violaxanthin to zeaxanthin) showed consistent gradients of pNPQ formation in comparison to the wild type (Ruban & Belgio, 2014; Ware et al. 2014). Moreover, it was previously reported the relationship between qPd, as a photoinhibition marker, and photosynthetic oxygen evolution in intact leaves of Arabidopsis. Thus, these evidences have supported the consistence of qPd as an indicative of photoinhibition and its derived parameter, pNPQ, as related to a photoprotective mechanism, possibly heat dissipation component of NPQ, qE (Ruban and Belgio, 2014; Ware et al., 2014; Carvalho et al., 2015; Giovagnetti and Ruban, 2015).

Our data concerning pNPQ and the mechanisms underlying NPQ formation during ontogenesis add a new step on the discussion of pNPQ interpretation as an indicative of a photoprotection mechanism. Indeed, young plants exhibited higher PsbS relative amount in comparison to the other studied ages (Fig. 1). The main role of PsbS protein in the process of

NPQ formation is, by binding to antenna proteins, to increase the sensitivity of the membrane to pH changes, producing a new state of aggregation of light harvesting complexes that allow the excess energy dissipation as heat (Funk et al., 1995; Li et al., 2002; Niyogi et al., 2005). Indeed, *Arabidopsis -npq4* (lacking PsbS protein), which exhibits strong impairment in qE formation, are able to revert this deficiency if supplied with an artificial ∆pH inductor, such as DAD (Johnson and Ruban, 2011). Such reports suggest that the PsbS protein plays a signal amplifier role in thylakoidal events that lead to qE formation (Johnson and Ruban, 2011). In this current study, juvenile plants exhibited the lowest potential of ∆pH among the studied plants (Fig. 2). Thus, these results indicate that the higher relative amount of PsbS presented by juvenile plants might be related to a compensatory mechanism to the low ∆pH formation capability displayed by these young plants.

Juvenile plants also exhibited higher content of zeaxanthin and violaxanthin deepoxidase activity, after exposure to 300 μmol m^{-2} s^{-1} for 45 min, in comparison to the older plants (Fig. 3). Under these light intensity, juvenile *Arabidopsis* plants exhibited strong indicatives of photoinhibition, while mature plants did not display photoinhibition as evidenced by previous qPd measures (Carvalho et al., 2015). Moreover, the total content of xanthophyll cycle carotenoids in mature plants was much higher than that exhibited by juvenile and senescent plants. Taken together these results clearly indicate that under light intensity as high as 300 μmol m^{-2} s^{-1}, juvenile *Arabidopsis* plants activated the defence mechanisms related to NPQ formation to its maximum. Since when mature several plant species present a trend to reach maximum CO_2 assimilation rate (Sestak and Catsky, 1962; Čatský and Tichá, 1979) as well as ETRII and FvFm (Siffel et al., 1993; Carvalho et al., 2015) it is possible that *Arabidopsis* plants at mature stage of development presented higher energetic demand, avoid excess energy and trigger defence mechanisms.

In this present study, mature plants exhibited the lowest β-carotenoid contents among the plants (Fig. 3). β-carotenoids are related to several different roles on plant chloroplasts,

since acting in energy harvesting, especially at blue light range (Harvaux and Kloppstech, 2001; Ramel et al., 2012), to its roles as antioxidant molecule (Davison et al., 2002; Ramel et al., 2012). *Arabidopsis* plants at juvenile and senescent phases of ontogenesis present higher indicatives of oxidative stress, as evidenced by increase in H_2O_2 accumulation and lipid peroxidation (Carvalho et al., 2015). Moreover, the anthocyanin content in *Arabidopsis* followed the same age-dependent pattern associated to oxidative stress symptoms (Carvalho et al., 2015). Thus is reasonable to propose that the higher β-carotenoid contents exhibited by *Arabidopsis* plants at juvenile and senescent phases, in comparison to mature phase, are associated to the roles of this pigment in antioxidative protection.

In mature plants, the PSII-LHCII super-complex relative amount and, especially, reaction centers core of photosystem II (RCII core) relative amount were much higher than that exhibited by juvenile and senescent plants (Fig. 5, Table 1). These data reinforce that mature phased plants encompass a significantly bigger apparatus to capture and process energy in thylakoids, which should be associated with chloroplast maturation (Avery and Avery Jr, 1933; Kutík et al., 1999) as well as whole plant increase of energetic demand (Čatský and Tichá, 1979). In contrast, the PSII/PSI ratios in senescent aged were higher than in mature and juvenile plants. This difference was consistent with the PSII/PSI activity, measured by 77 °K fluorescence (Fig. 5, Table 2). Higher PSII/PSI rates could suggest a preliminary evidence for a reduced capability of cyclic electron flux (CEF) generation/functioning (Yamori et al., 2011). CEF is an important photoprotection mechanism in plants subjected to unbalance between light energy capture and energetic demand, as occur during high light stress (Takahashi et al., 2009). CEF is especially related to the maintenance of ΔpH formation between lumen and stroma under excess energy conditions (Takahashi et al., 2009), which in turn is an essential requirement for NPQ formation (Demmig-Adams and Adams, 1992; Ruban et al., 1992). Thus in combination with reduced RCII core relative amount, reduced CEF could explain, at least in part, the lower light tolerance of senescent plants in comparison to the reported for mature phase.

Taken together, our data evidenced that both juvenile and senescent plants were restricted in important mechanisms related to NPQ formation. Thus, it is reasonable to suggest that previous quantified differences in pNPQ formation are possibly dependent on the balance between PsbS content, ∆pH accumulation and violaxanthin/zeaxanthin de-epoxidase activity and other parallel photoprotective mechanisms, such as CEF and antioxidant apparatus. Nevertheless, the mature plants exhibited the most advantageous combination of NPQ underlying mechanisms. This feature exhibited by these plants was probably important to the most efficient pNPQ formation and subsequently higher light intensity tolerance, previously observed.

Acknowledgments

AVR would like to thank UK BBSRC and The Leverhulme Trust for the financial support. Fabricio E. L. Carvalho is supported by the Science Without Boarders program (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq – Brazil)/INCT-Sal. FELC is grateful to Prof. José Tadeu Oliveira for English writing review.

References

Ahn TK, Avenson TJ, Ballottari M, Cheng Y-C, Niyogi KK, Bassi R, Fleming GR (2008) Architecture of a charge-transfer state regulating light harvesting in a plant antenna protein. Science **320**: 794–797

Amasino R (2010) Seasonal and developmental timing of flowering. Plant J **61**: 1001–1013

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55**: 373–399

Arnon DI, Allen MB, Whatley FR (1954) Photosynthesis by isolated chloroplasts. Nature **174**: 394–396

Asada K (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol **141**: 391–396

Avery GSJ, Avery Jr G (1933) Structure and development of the tobacco leaf. Am J Bot **20**: 565–592

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu Rev Plant Biol **59**: 89–113

Betterle N, Ballottari M, Zorzan S, de Bianchi S, Cazzaniga S, Dall'Osto L, Morosinotto T, Bassi R (2009) Light-induced dissociation of an antenna hetero-oligomer is needed for non-photochemical quenching induction. J Biol Chem **284**: 15255–15266

Boyes DC, Zayed AM, Ascenzi R, McCaskill AJ, Hoffman NE, Davis KR, Görlach J (2001) Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. Plant Cell **13**: 1499–1510

Carvalho FEL, Ware MA, Ruban AV (2015) Quantifying the dynamics of light tolerance in Arabidopsis plants during ontogenesis. Plant Cell Environ **38**: 2603–2617

Čatský J, Tichá I (1979) CO_2 compensation concentration in bean leaves: Effect of photon flux density and leaf age. Biol Plant **21**: 361–364

Davison PA, Hunter CN, Horton P (2002) Overexpression of beta-carotene hydroxylase enhances stress tolerance in Arabidopsis. Nature **418**: 203–206

Demmig-Adams B, Adams WW (1992) Photoprotection and other responses of plants to high light stress. Annu Rev Plant Physiol Plant Mol Biol **43**: 599–626

Farber A, Young AJ, Ruban AV, Horton P, Jahns P (1997) Dynamics of xanthophyll-cycle activity in different antenna subcomplexes in the photosynthetic membranes of higher plants (the relationship between zeaxanthin conversion and nonphotochemical fluorescence quenching). Plant Physiol **115**: 1609–1618

Funk C, Schröder WP, Napiwotzki A, Tjus SE, Renger G, Andersson B (1995) The PSII-S protein of higher plants: A new type of pigment- binding protein. Biochemistry **34**: 11133–11141

Giovagnetti V, Ruban AV (2015) Discerning the effects of photoinhibition and photoprotection on the rate of oxygen evolution in Arabidopsis leaves. J Photochem Photobiol B Biol **152**: 272-278

Harvaux M, Kloppstech K (2001) The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in Arabidopsis npq and tt mutants. Planta **213**: 953–966

Hill R, Bendall F (1960) Function of the two cytochrome components in chloroplasts: a working hypothesis. Nature **186**: 136–137

Hopkinson JM (1964) Studies on the expansion of the leaf surface - IV The carbon and phosphorous economy of a leaf. JExpBot **15**: 125–137

Horton P, Ruban AV, Walters RG (1996) Regulation of light harvesting in green plants. Annu Rev Plant Physiol Plant Mol Biol **47**: 655–684

Johnson MP, Ruban AV (2011) Restoration of rapidly reversible photoprotective energy dissipation in the absence of PsbS protein by enhanced DeltapH. J Biol Chem **286**: 19973–19981

Kerstetter RA, Poethig RS (1998) The specification of leaf identity during shoot development. Annu Rev Cell Dev Biol **14**: 373–398

Kozuka T, Horiguchi G, Kim GT, Ohgishi M, Sakai T, Tsukaya H (2005) The different growth responses of the *Arabidopsis thaliana* leaf blade and the petiole during shade avoidance are regulated by photoreceptors and sugar. Plant Cell Physiol **46**: 213–223

Krause GH, Behrend U (1986) ΔpH-dependent chlorophyll fluorescence quenching indicating a mechanism of protection against photoinhibition of chloroplasts. FEBS Lett **200**: 298–302

Kutík J, Kočová M, Holá D, Körnerová M (1999) The development of chloroplast ultrastructure and Hill reaction activity during leaf ontogeny in different *maize (Zea mays* L.) genotypes. Photosynthetica **36**: 497–507

Li X-P, Muller-Moule P, Gilmore AM, Niyogi KK (2002) PsbS-dependent enhancement of feedback de-excitation protects photosystem II from photoinhibition. Proc Natl Acad Sci USA **99**: 15222–15227

Matile P, Schellenberg M, Peisker C (1992) Production and release of a chlorophyll catabolite in isolated senescent chloroplasts. Planta **187**: 230–235

Milthorpe FL (1959) Studies on the expansion of the leaf surface I - The influence of temperature. JexpBot **10**: 233–249

Nath K, Phee BK, Jeong S, Lee SY, Tateno Y, Allakhverdiev SI, Lee CH, Nam HG (2013) Age-dependent changes in the functions and compositions of photosynthetic complexes in the thylakoid membranes of *Arabidopsis thaliana*. Photosynth Res **117**: 547–556

Niyogi KK, Li X-P, Rosenberg V, Jung H-S (2005) Is PsbS the site of non-photochemical quenching in photosynthesis? J Exp Bot **56**: 375–382

Paul MJ, Foyer CH (2001) Sink regulation of photosynthesis. J Exp Bot **52**: 1383–1400

Porra RJR, Thompson WWA, Kriedemann PPE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: Biochim Biophys Acta **975**: 384–394

Ramel F, Birtic S, Ginies C, Soubigou-Taconnat L, Triantaphylides C, Havaux M (2012) Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. Proc Natl Acad Sci USA **109**: 5535–5540

Raven JA (1984) Physiological correlates of the morphology of early vascular plants. Bot J Linn Soc **88**: 105–126

Ruban AV (2014) Evolution under the sun: optimizing light harvesting in photosynthesis. J Exp Bot **66**: 7–23

Ruban AV, Belgio E (2014) The relationship between maximum tolerated light intensity and photoprotective energy dissipation in the photosynthetic antenna: chloroplast gains and losses. Philos Trans R Soc B Biol Sci **369**: 20130222–20130222

Ruban AV, Murchie EH (2012) Assessing the photoprotective effectiveness of non-photochemical chlorophyll fluorescence quenching: A new approach. Biochim Biophys Acta - Bioenerg **1817**: 977–982

Ruban AV, Rees D, Pascal AA, Horton P (1992) Mechanism of ΔpH-dependent dissipation of absorbed excitation energy by photosynthetic membranes. II. The relationship between LHCII aggregation in vitro and qE in isolated thylakoids. Biochim Biophys Acta - Bioenerg **1102**: 39–44

Ruban AV, Lee PJ, Wentworth M, Young AJ, Horton P (1999) Determination of the stoichiometry and strength of binding of xanthophylls to the photosystem II light harvesting complexes. J Biol Chem **274**: 10458–10465

Ruban AV, Berera R, Ilioaia C, van Stokkum IHM, Kennis JTM, Pascal AA, van Amerongen H, Robert B, Horton P, van Grondelle R (2007) Identification of a mechanism of photoprotective energy dissipation in higher plants. Nature **450**: 575–578

Ruban AV, Johnson MP, Duffy CDP (2012) The photoprotective molecular switch in the photosystem II antenna. Biochim Biophys Acta - Bioenerg **1817**: 167–181

Ruban AV, Solovieva S, Lee PJ, Ilioaia C, Wentworth M, Ganeteg U, Klimmek F, Wah SC, Anderson JM, Jansson S, et al (2006) Plasticity in the composition of the light harvesting antenna of higher plants preserves structural integrity and biological function. J Biol Chem **281**: 14981–14990

Schuldiner S, Rottenberg H, Avron M (1972) Determination of pH in chloroplasts. 2. Fluorescent amines as a probe for the determination of pH in chloroplasts. Eur J Biochem **25**: 64–70

Sestak Z, Catsky J (1962) Intensity of photosynthesis and chlorophyll content as related to leaf age in *Nicotiana sanderae* Hort. Biol Plant **4**: 131–140

Siffel P, Santrucek J, Lang M, Braunova Z, Simkova M, Synkova H, Lichtenthaler HK (1993) Age dependence of photosynthetic activity, chlorophyll fluorescence parameters and chloroplast ultrastructure in aurea and green forms of *Nicotiana tabacum* Su/su mutant. Photosynthetica **29**: 81–94

Takahashi S, Badger MR (2011) Photoprotection in plants: A new light on photosystem II damage. Trends Plant Sci **16**: 53–60

Takahashi S, Milward SE, Fan D-Y, Chow WS, Badger MR (2009) How does cyclic electron flow alleviate photoinhibition in Arabidopsis? Plant Physiol **149**: 1560–1567

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76**: 4350–4354

Tsukaya H (2002) Leaf development. Arab B **1**: e0072

Ware MA, Giovagnetti V, Belgio E, Ruban AV (2015) PsbS protein modulates non-photochemical chlorophyll fluorescence quenching in membranes depleted of photosystems. J Photochem Photobiol B Biol **152**:301–307

Ware MA, Belgio E, Ruban AV (2014) Comparison of the protective effectiveness of NPQ in Arabidopsis plants deficient in PsbS protein and zeaxanthin. J Exp Bot **66**: 1259–1270

Yamori W, Sakata N, Suzuki Y, Shikanai T, Makino A (2011) Cyclic electron flow around photosystem I via chloroplast NAD(P)H dehydrogenase (NDH) complex performs a significant physiological role during photosynthesis and plant growth at low temperature in rice. Plant J **68**: 966–976

Zulfugarov IS, Tovuu A, Eu Y-J, Dogsom B, Poudyal R, Nath K, Hall M, Banerjee M, Yoon U, Moon Y-H, et al (2014) Production of superoxide from photosystem II in a rice (*Oryza sativa* L.) mutant lacking PsbS. BMC Plant Biol **14**: 242

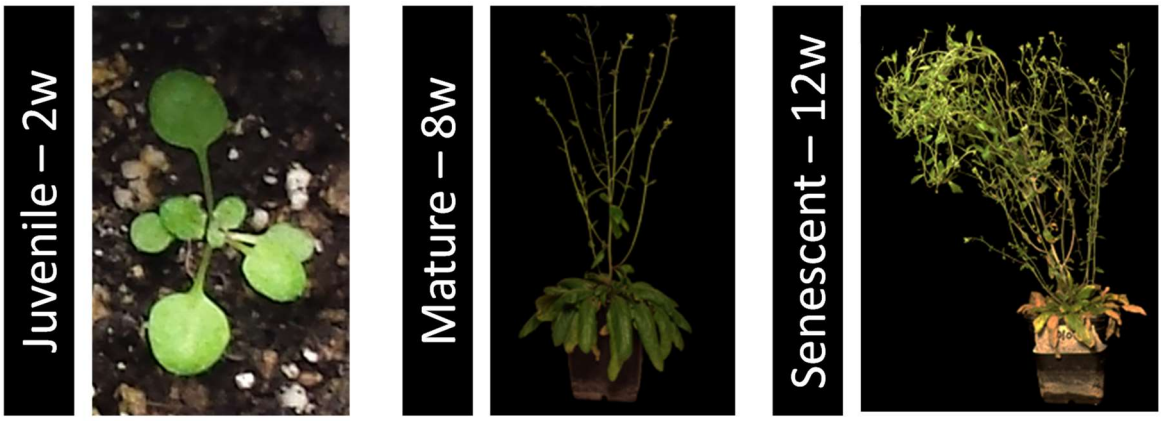

Figure 1. Visual aspect of *Arabidopsis thaliana* at juvenile, mature and senescent phases. The juvenile phase was represented by plants aged 2 weeks old. The mature phase was represented by plants aged 8 weeks old. The senescent phase was represented by plants aged 12 weeks old. Figures are the most representative of 10 independent plants.

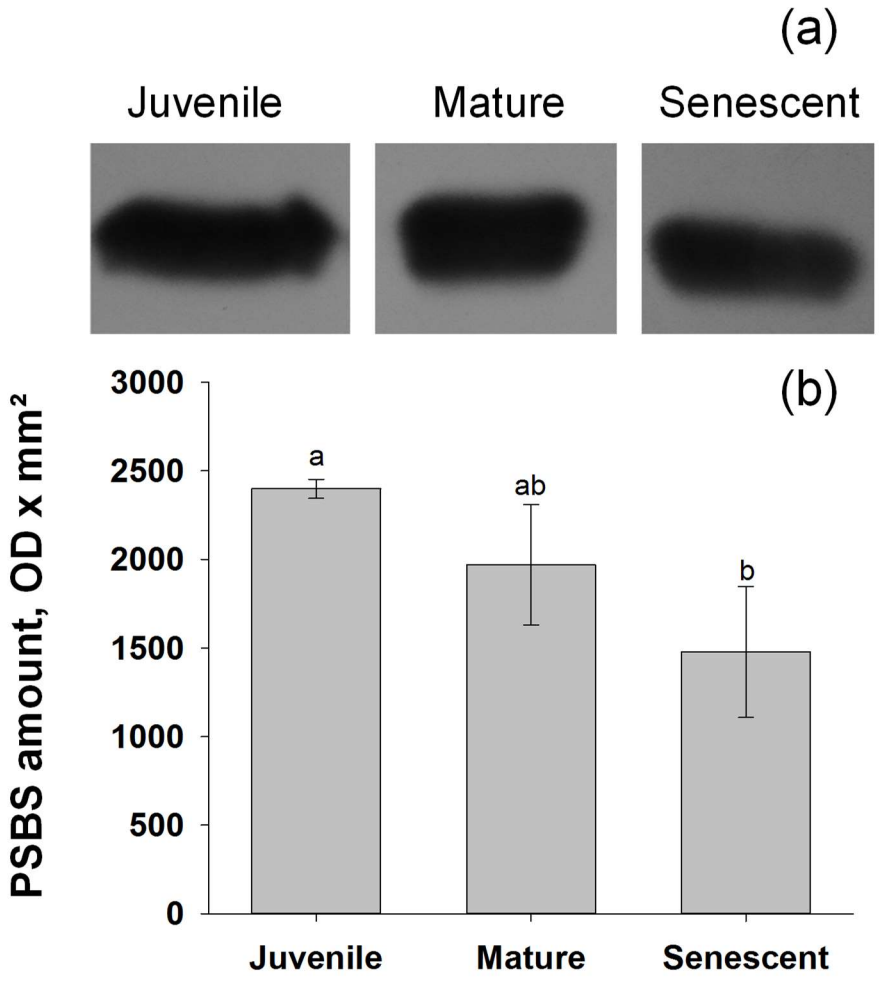

Figure 2. (a) Western blotting of PsbS protein from leaves of *Arabidopsis thaliana* plants at juvenile, mature and senescent phases of ontogenesis. 1.2 µg of total chlorophyll was loaded in each lane from total leaf protein extracts, which was obtained from 5 independent leaves each. (b) Quantification of PsbS protein amount obtained from optical density (OD) evaluation using the software Image Master 2.0 (Amersham Biosciences). Error bars represent SEM (n=5) Different letters represent significant difference by student t test, $p \leq 0.05$.

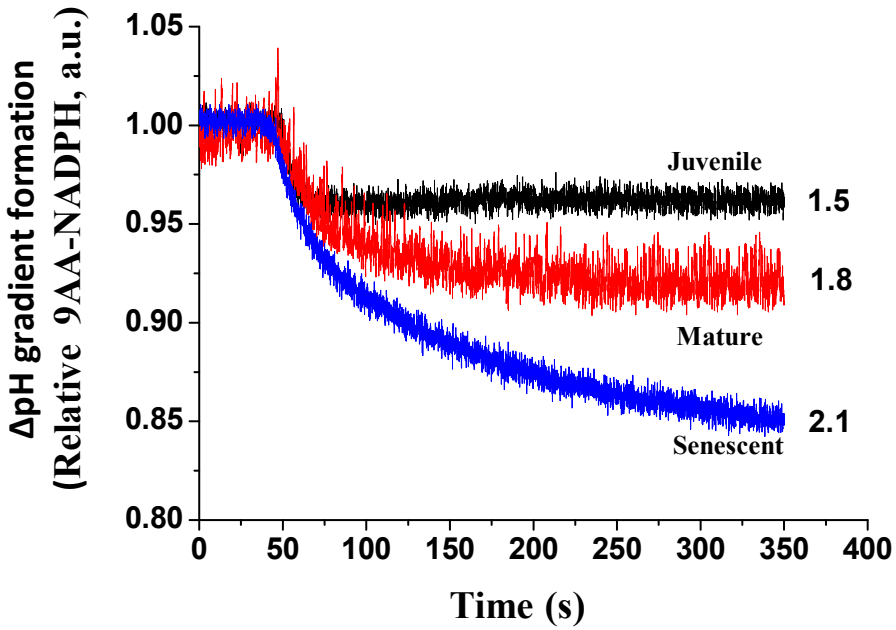

Figure 3. ΔpH gradient formation measured in isolated chloroplasts from leaves of *Arabidopsis thaliana* plants at juvenile, mature and senescent phases of ontogenesis. 18 µg of total chlorophyll was loaded for each measure, which was obtained from 3 independent plants at each phase. For each test 2 µL of DAD (0.15 M), 3 µL of methyl viologen (0.1 M) and 15 µL of 9aa (1 mM) were used. The actinic light equals to 2,000 µmol m^{-2} s^{-1}.

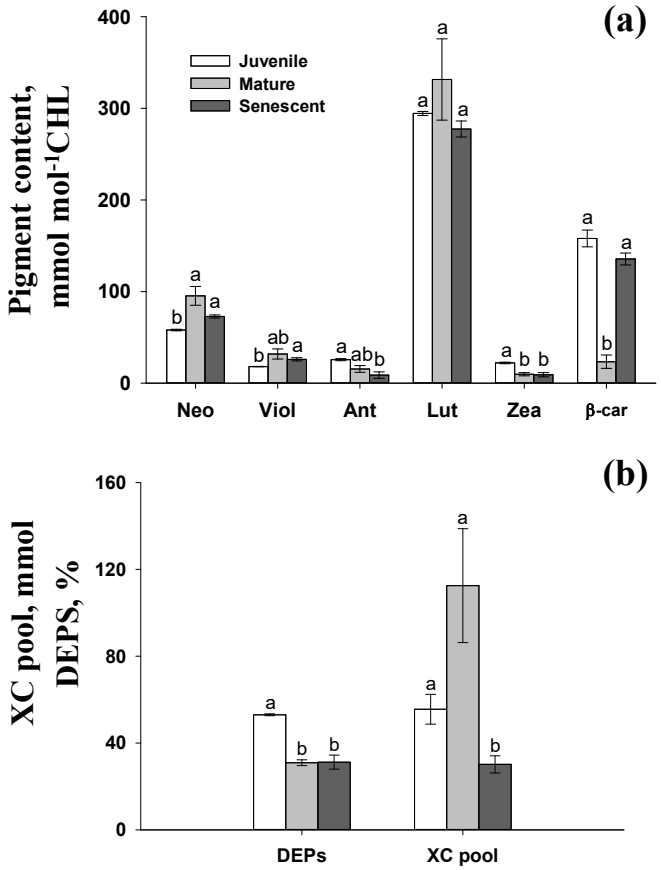

Figure 4. Carotenoids profile quantified by HPLC in extracts from leaves of *Arabidopsis thaliana* plants at juvenile, mature and senescent phases of ontogenesis. (a) Contents of neoxanthin (neo), violaxanthin (viol), anteroxanthin (ant), lutein (lut), zeaxanthin (zea) and β-carotene (β-car). The values are expressed in mmol per mol of total chlorophyll. (b) The de-epoxidation state (%) and the xanthophylls cycle pool (mmol). The leaves were previously illuminated – 380 μmol m^{-2} s^{-1}, for 45 min, in the presence of saturating N_2. Error bars represent SEM (n=3) Different letters represent significant difference by student t test, $p \leq 0.05$.

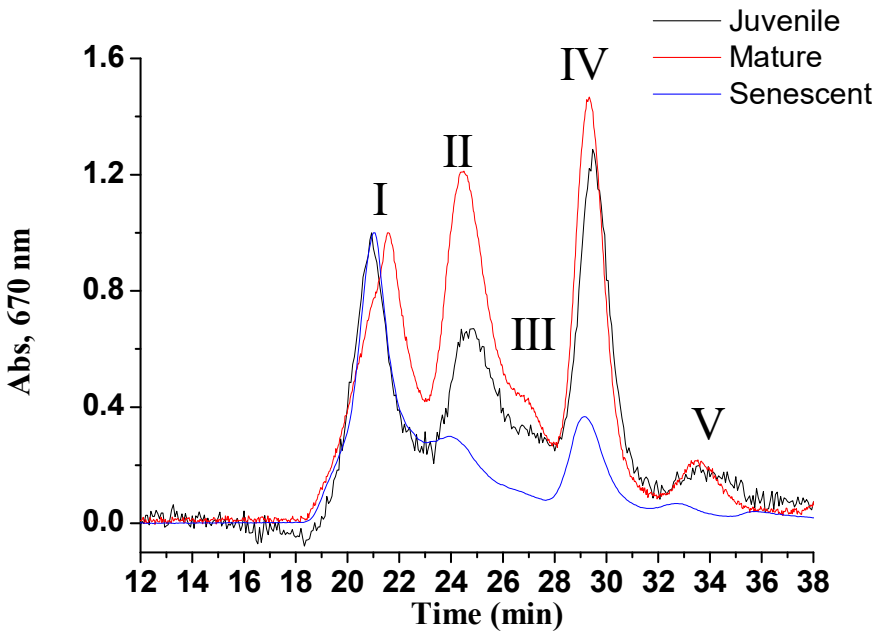

Figure 5. Photosynthetic super-complexes ratios measured in stacked thylakoids enriched fraction, obtained from leaves of *Arabidopsis thaliana* plants at juvenile, mature and senescent phases of ontogenesis. α-DM was added to each sample to increase the solubilization in a proportion of 1% (v:v) per mg of total chlorophyll per mL. I – Thylakoid membrane fragments, II – PSII-LHCII super-complexes, III – PSI, IV – PSII core complexes, V – LHCII trimers.

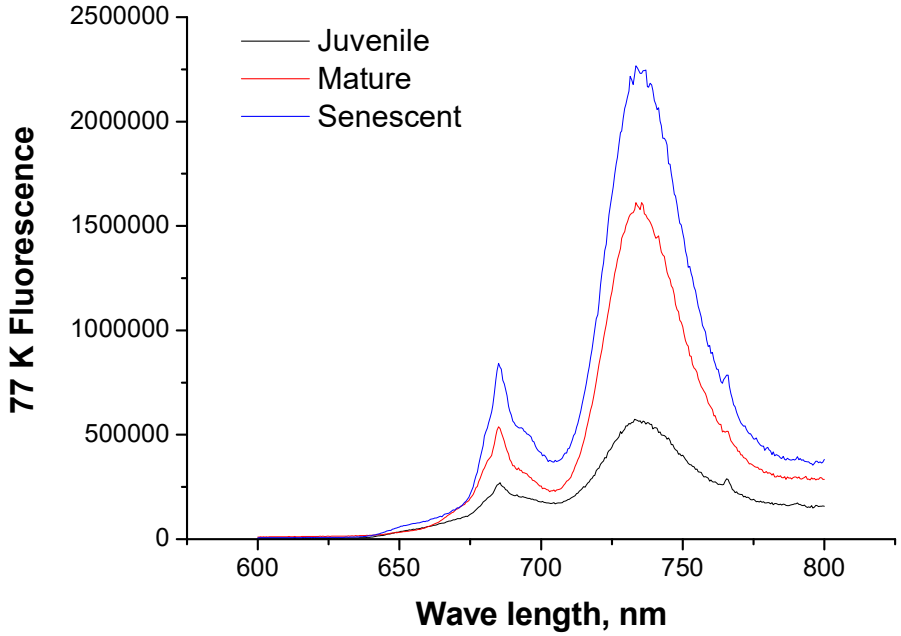

Figure 6. 77 °K and fluorescence emission spectra measured on thylakoids extracts from *Arabidopsis thaliana* plants at juvenile, mature and senescent phases of ontogenesis. The thylakoid membranes isolated were subsequently injected into the sample holder and shock-frozen in liquid N_2. Fluorescence spectral resolution was set as 1 nm, with 5 nm of spectral bandwidth and excitation defined as 435 nm.

Table 1. Peaks obtained by FPLC separation spectra at 670 nm from plants at juvenile, mature and senescent phases of ontogenesis.

Peak	Complex	Gaussian peak area		
		Juvenile	Mature	Senescent
1	PSII-LHCII supercomplexes	1.65	1.82	1.56
2	PSI	1.78	2.10	0.52
3	PSII Core	0.41	1.30	0.39
4	LHCII trimers	1.62	1.67	0.44
5	Monomeric LHCII	0.38	0.35	0.08
	Ratio			
	PSII/PSI	0.23	0.62	0.75
	Trimers/PSII	3.96	1.28	1.12

Table 2. Comparison between PSII and PSI 77 °K fluorescence emission peaks measured on thylakoids extracts from juvenile, mature and senescent *Arabidopsis thaliana* plants.

Plant Age	PSII fluorescence	PSI fluorescence	PSII/PSI fluorescence ratio
Juvenile	841461.1	2267110	0.37
Mature	538136.6	1612270	0.33
Senescent	270450.8	574035.9	0.47

INTERCHAPTER

Data displayed in chapter II of the current study have evidenced the successfully use of fluorimetric-based methodology for qPd determination in the model plant *A. thaliana*, during ontogenesis and the importance of chlorophylls and antioxidative metabolism for photoprotection. Moreover, chapter III suggests mechanisms underlying pNPQ formation differences during ontogenesis are complex and dependent of a specific combination in ∆pH levels, violaxanthin/zeaxanthin ratios and PsbS protein amounts. Moreover, the total xanthophyll pool was higher in mature Arabidopsis plants, coinciding with maximum light tolerance during ontogenesis. These evidences reinforce the importance of photosynthetic pigments for pNPQ formation efficiency and photoprotection. In order to test qPd-based methodology in crop models, experiments involving *Oryza sativa* plants were designed. In addition, aiming to investigate the importance of ROS in pNPQ formation and light tolerance, rice plants silenced by RNAi for thylakoidal APX (*apx8*) were obtained. Chloroplast APX are responsible for 20% of total APX activity in plant cells, and among them, thylakoidal isoforms are believed to display great importance in Arabidopsis. However, several studies involving deficient mutants have reported intriguing and contradictory results regarding importance of these enzymes, such as no differences in Fv/Fm and absence of phenotype. In the next chapter are described the main results obtained involving the photoinhibition onset in *apx8* plants exposed to different light regimes.

CHAPTER IV

Fabricio E.L. Carvalho

(Unpublished data – Manuscript draft)

Original research

Thylakoidal APX knockdown regulates NPQ formation under moderate light conditions in rice plants

Fabricio E.L Carvalho[1], Juliana R. Cunha[1], Alexander V. Ruban[2], Marcio O. Martins[1], João Víctor A. Cerqueira[1], Marcia Margis-Pinheiro[3] and Joaquim A.G. Silveira[1]*

[1]Department of Biochemistry and Molecular Biology, Federal University of Ceara, Fortaleza/CE – Brazil, Zip Code 60455-970.

[2]School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK

[3]Department of Genetics, Federal University of Rio Grande do Sul, Porto Alegre/RS - Brazil, Zip Code 91501-970.

Corresponding author

Prof. J.A.G. Silveira; Departamento de Bioquímica e Biologia Molecular, Laboratório de Metabolismo de Plantas, Universidade Federal do Ceará. Av. Humberto Monte SN, Campus do Pici, Bl. 907. Fortaleza, CEP 60451-970, Ceará, Brasil. Phone: +55 85 3366 9821. E-mail: silveira@ufc.br

Abstract – Chloroplastic ascorbate peroxidases are the first described APX in literature and responsible for 20% of total APX activity in plant cells. Surprisingly, plants deficient in thylakoidal APX (tAPX) did not exhibit phenotypic changes under moderate light and unaltered tolerance to high light. Employing tAPX knockdown rice (*apx8*) as model and the new fluorimetric methodologies for photoinhibition quantification based on dark-measured photochemical quenching (qPd), the current work aimed to unveil the concrete physiological importance of tAPX for plant defence responses under excessive light conditions. Under moderate light, *apx8* plants exhibit slightly stunted growth and impaired seed production in comparison to non-transformed plants (NT). Our data clearly shows that *apx8* plants present higher susceptibility to light stress in comparison to NT. Moreover, the reduced light tolerance of *apx8* plants is explained, at least in part, by an impairment in NPQ formation capability. Nevertheless, ΔpH formation and violaxanthin deepoxidase activity possibly were less involved in this NPQ restriction, since *apx8* was similarly susceptible to DTT or Nigericin infiltration in comparison to NT. Increased H_2O_2 levels and decreased chlorophyll content are probably important features related with NPQ deficiency in *apx8* plants. Interestingly, under long-term excessive light conditions, compensatory peroxidase mechanisms are triggered in *apx8* plants, NPQ formation capability is recovered and silenced plants are able to acclimate to such conditions as well as NT. Therefore, these evidences suggest a conditional importance of tAPX in rice plants, restricted to moderate light conditions, related to H_2O_2, total chlorophyll and NPQ levels.

Keywords: Chloroplastic APX, High light, NPQ, *Oryza sativa*, Photoinhibition.

Introduction

Ascorbate peroxidase (APX, EC. 1.11.1.11) is a very important enzyme to maintenance of hydrogen peroxide (H_2O_2) levels in plant cells (Nakano and Asada, 1981). Indeed, APX in higher plants are encoded by small multigene family and different isoforms are classified according to their subcellular localization (Teixeira et al., 2004; Teixeira et al., 2006). In rice (*Oryza sativa* L.), eight different APX isoforms have been characterized to date: *OsAPX1* and *OsAPX2* (cytosol), *OsAPX3* and *OsAPX4* (peroxisome), *OsAPX5* (mitochondria/chloroplast), *OsAPX6* (mitochondria), *OsAPX7* (stroma) and *OsAPX8* (thylakoid membranes) - (Teixeira et al., 2004; Teixeira et al., 2006). In parallel to catalase (CAT, 1.11.1.6), APX have been for a long time considered among the main enzymes related to H_2O_2 scavenging, but differently from CAT, APX presents high affinity for H_2O_2 (Yamaguchi et al., 1995). Therefore, APX is believed to act in H_2O_2 fining tuning and, since this molecule is believed to be an important abiotic stress-signalling (Sewelam et al., 2014), the possible importance of APX working on an antioxidative hub in plant cells have been intensely discussed in the last years (Bonifacio et al., 2011; Shigeoka and Maruta, 2014; Maruta et al., 2016).

Among APX isoenzymes, the cytosolic isoforms are considered to be very important (Amako et al., 1994) and, therefore, most of studies have been focused on the importance of these isoforms for plant antioxidative defence (Pnueli et al., 2003; Davletova et al., 2005; Koussevitzky et al., 2008; Maruta et al., 2012). These studies are mainly limited to *Arabidopsis thaliana* L. and *Nicotiana tabacum* L. as plant models. Chloroplastic APX have been first described in literature (Nakano and Asada, 1981), and some studies have focused on the roles of these enzymes for antioxidative protection in plant cells so far (Neubauer and Yamamoto, 1992; Neubauer and Yamamoto, 1994; Mano et al., 2001; Giacomelli et al., 2007; Bechtold et al., 2008; Maruta et al., 2010; Caverzan et al., 2014). Indeed, chloroplastic APX are believed to be responsible for 20% of total APX activity in plant cells (Amako et al., 1994).

In leaves of higher plants, chloroplasts are a very important site of H_2O_2 generation (Foyer and Noctor, 2003). The production of oxygen reactive species (ROS) such as superoxide radical ($O_2^{\bullet-}$) is a natural consequence of energy flow in thylakoid membranes (Ogawa et al., 1997). Actually, both PSI and PSII could act as $O_2^{\bullet-}$ formation site in chloroplasts (Ogawa et al., 1997; Zulfugarov et al., 2014). Regardless $O_2^{\bullet-}$ generation site, this molecule is rapidly scavenged by superoxide dismutase (SOD, EC 1.15.1.1) and converted to H_2O_2 (Pilon et al., 2011). Under abiotic stress, an unbalance between the amount of light captured by light harvest complex antennas (LHC) and the energy demanded by metabolic reactions (especially CO_2 assimilation) is a very common condition observed in plants. This condition is commonly associated to the over-accumulation of H_2O_2 and other ROS in leaf tissues, occasionally generating photoinhibition and even plant death (Goh et al., 2012).

Chloroplast APX are essential enzymes to avoid H_2O_2 over-accumulation, which is related to the impairment of D1 protein *de novo* synthesis, since this molecule affects the elongation factor G, leading to photoinhibition (Murata et al., 2012). Moreover, acting in water-water cycle, chloroplast APX are believed to be able to sustain linear electron flow in thylakoids membranes, inducing ΔpH formation and, therefore, non-photochemical quenching (NPQ) - (Neubauer and Yamamoto, 1992). The major and fastest component of NPQ, qE, is believed to act in PSII reaction centre (RCII) photoprotection, and is related to the dissipation of excess energy as heat (Ruban, 2016). The site of qE formation is probably related to PSII antennas (Chmeliov et al., 2015; Ruban, 2016), triggered by ΔpH and enhanced by violaxanthin de-epoxidation to zeaxanthin (Demmig-Adams and Adams, 1992; Ruban et al., 1992). In addition, PsbS protein is believed to play a crucial role in sensing lumen acidification and transducing the signal to the PSII antennas (Funk et al., 1995; Li et al., 2000; Niyogi et al., 2005).

Despite the possible involvement of chloroplastic APX in these important photoprotective mechanisms, studies regarding *A. thaliana* transformed plants deficient in stromal and thylakoidal APX isoforms have reported a minor importance of these enzymes in

plant stress protection (Giacomelli et al., 2007; Kangasjärvi et al., 2008; Maruta et al., 2010). These studies revealed that both thylakoidal APX (*tapx*) and stromal APX (*sapx*) deficient plants do not display any visible phenotypic alterations under non-stressful growth conditions. Nevertheless, under photooxidative stress, *tapx* mutants exhibited marked effect on the accumulation of H_2O_2 and oxidized protein, rather than the *sapx* plants (Maruta et al., 2010).

The higher susceptibility of *tapx* plants to photooxidative stress in comparison to NT is apparently limited to the first hours of light stress imposition (Maruta et al., 2010), it is dependent on levels of ascorbate – ASC (Giacomelli et al., 2007) and it is possibly compensated under long term stress by 2-cys-peroxiredoxins (Kangasjärvi et al., 2008). Interestingly, chlAPX double knockdown rice (*apx7/8*) does not display any phenotypic changes under normal growth conditions and is also capable to cope with high light stress as well as NT plants (Caverzan et al., 2014). Indeed, *apx7/8* exhibited higher impairment in Fv/Fm than NT plants only when exposed to methylviologen – MV (Caverzan et al., 2014). Whether thylakoidal APX is important for light tolerance or not and which biochemical mechanisms could be involved with this enzyme protective role during excessive light stress are issues that remain to be clarified.

In the current study, we evidenced that tAPX knockdown rice plants (*apx8*) exhibit slightly stunted growth and impaired seed production in comparison to NT. In order to reveal the events underlying this phenotype, we employed a new methodology of photoinhibition tracking by qPd determination (Ruban and Murchie, 2012). Our data clearly shows that *apx8* plants present higher susceptibility to light stress in comparison to NT. Moreover, the reduced light tolerance of *apx8* plants is explained, at least in part, by an impairment in NPQ formation. Surprisingly, transformed plants did not exhibit changes in ETRII in response to light curve and displayed similar susceptibility to NPQ inhibition by nigericin. In addition, *apx8* plants did not showed changes in total ascorbate levels, ASC redox state, and suffered similar NPQ inhibition induced by DTT. Therefore, ΔpH and violaxanthin deepoxidase activity are probably less

involved in NPQ restriction observed in these mutants. In contrast, increased H_2O_2 levels and decreased chlorophyll content are probably important features related with NPQ deficiency in *apx8* plants. The physiological importance of thylakoidal APX proteins for light tolerance in rice plants is discussed.

Material and methods

Construction of plant vectors and plant transformation

In order to generate *apx8* silenced rice plants, a 227 bp sequence was amplified by PCR based on the sequence of the *OsAPX8* gene (LOC_Os02g34810). The primers used were 5'-CTCGAGGCTGCGAAATACTCCTACGG-3' and 5'-GGTACCTCGAGAGGAGGTCATCAGACCATCG-3'. The PCR product was cloned into the pANDA vector (Miki and Shimamoto, 2004), which enables hairpin structure formation and post-transcriptional silencing of the *OsAPX8* gene. The pANDA vector contains the maize ubiquitin promoter and the *Hpt* gene for selection by hygromycin. The transformation of rice calli was achieved via *Agrobacterium tumefaciens*, as described previously (Upadhyaya et al., 2000). Regenerated seedlings were grown at 28 °C in MS medium with a photoperiod of 12 h and 150 µmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD) in a growth chamber for seven days. Five lines of *apx8*-transformed plants were selected to phenotypic characterization.

Plant growth

Among transformed plant lines *apx8* L7 presented greater impairment in biomass accumulation and seed productivity (Fig. S1). Thus, based on these phenotypic features, the *apx8* L7 line was selected for further experiments. Silenced *apx8* and the NT plants (T2

generation) were transferred to 2 L plastic pots filled with half-strength Hoagland's solution. The pH was adjusted to 6.0 ± 0.5 every two days, and the nutrient solution was changed weekly. The seedlings were previously grown for 30 days in a greenhouse with the following conditions: day/night mean temperature of 29/24 °C, mean relative humidity of 68%, and a photoperiod of 12 h. The light intensity inside the greenhouse varied as for a typical day from 6:00 a.m. to 6:00 p.m., reaching an average maximum PPFD of 820 μmol m^{-2} s^{-1} at noon. Subsequently, plants were transferred to a growth chamber at 5-10 days before the beginning of the experiments. The growth chamber was set with the following conditions: day/night mean temperature of 29/24 °C, mean relative humidity of 70%, a photoperiod of 12 h and PPFD of 400 μmol m^{-2} s^{-1}.

High light stress experiments

Silenced *apx8* and the NT plants (T2 generation) ageing 35-45 days (acclimated to growth chamber conditions) were exposed to different light regimes. The control group remained under PPFD of 400 μmol m^{-2} s^{-1} as described above. High light stress group (HL), was exposed to PPFD of 1800 μmol m^{-2} s^{-1} by four days. During this experiment, the photoperiod was kept on 12 h day/night. Alternatively, a second experiment was conducted employing continuous light. For such experiments, a group of plants (NT and *apx8*) remained at PPFD of 400 μmol m^{-2} s^{-1} continuously for 24 hours. Other group of plants was subjected to PPFD of 2000 μmol m^{-2} s^{-1} continuously for 24 hours. At the end of each experiment, chlorophyll *a* fluorescence and infrared gas analyses were performed as described below. In addition, leaf samples were collected, rapidly frozen with liquid N_2 for biochemical assays.

Leaf segments vacuum-infiltration experiments

Silenced *apx8* and the NT plants (T2 generation) ageing 35-45 days (acclimated to growth chamber conditions) were exposed to PPFD of 400 μmol m^{-2} s^{-1} as described above. Subsequently, leaf segments with 5 cm length were collected from ~30 min dark-acclimated rice plants. The leaf segments were vacuum-infiltrated with mock, DTT or Nigericin solutions using a syringe. The mock solution consisted of 10 mM HEPES at pH 6.5, containing 1.5 mM $CaCl_2$ and 0.01% Triton X-100 (v/v). DTT solution consisted of mock solution added by 5 mM dithiothreitol (DTT). Nigericin solution consisted of mock solution added by 4 μM nigericin (Nig). After infiltration, leaf segments were kept in a petri dish containing the respective treatment solution (mock, DTT or Nig) for 5 min in the dark. Subsequently, chlorophyll *a* fluorescence measures were performed as described below.

Chlorophyll a fluorescence measurement

The chlorophyll *a* fluorescence measurements were performed as described previously (Ruban and Murchie, 2012; Carvalho et al., 2015). NT and *apx8* plants aged between 35-45 days were dark-adapted for 45 min. The measures were obtained with a DUAL-PAM 100 fluorimeter (Walz, Effeltrich, Germany), using a procedure of ~42 min. Approximately 30 different plants were subjected to three different sets of qPd determination procedures of one of the following actinic light regimes: 0, 100, 200, 400, 800, 1200, 1500, 2000 μmol m^{-2} s^{-1}; 0, 150, 300, 600, 700, 1000, 1300, 1800 μmol m^{-2} s^{-1}; or 0, 77, 250, 500, 750, 900, 1150, 1650 μmol m^{-2} s^{-1}. Each procedure was performed 8 times for qPd measurements. The procedures started with the following scheme: (ML on/FR on)–(30 s)–(SP)–(30 s)–(SP)–(5 s), where ML = measuring light, FR = far red light; SP = saturating pulse (6,000 μmol m^{-2} s^{-1} for 0.6 sec). PSII maximum quantum efficiency (Fv/Fm, where Fv = Fm-Fo) was determined with the first SP. Subsequently, for light measurements, the following scheme was employed: (AL on/FR off)–(120 s)–(SP)–(180 s)–(SP)–(AL off), where AL = actinic light. The NPQ [NPQ =

(Fm/Fm') -1] and ETR [ETR = actual quantum yield x PPFD x 0.84 x 0.5] values were calculated from the second SP. qPd was measured suddenly after actinic light was turned off. For qPd measurements, the following scheme was employed: (FR on)–(10 s)–(SP)–(5 s)–(AL on/FR off). The procedures were repeated with increasing AL intensities (Ruban and Murchie, 2012; Ruban and Belgio, 2014; Ware et al., 2014; Carvalho et al., 2015).

Gas exchange assays

The CO_2 assimilation rate (P_N), stomatal conductance (g_S), intercellular CO_2 partial pressure (*Ci*) and transpiration (*E*) were measured in fully expanded leaves from both non-transformed and *apx8* knockdown rice plants with a portable infrared gas analyser system equipped with an LED source and a leaf chamber (IRGA LI-6400XT, LI-COR, Lincoln, USA). The internal parameters in the IRGA chamber during gas exchange measurements were 1000 μmol m^{-2} s^{-1} PPFD, 1.0 ± 0.2 kPa VPD and 38 Pa CO_2, at 28 °C. The amount of blue light was set to 10% of the PPFD to maximize the stomatal aperture (Flexas et al., 2008). The P_N was measured in response to changes in Ci. Each of these conditions was separately controlled inside the IRGA leaf chamber. The P_N-Ci fitting curves were determined according to models proposed previously in literature (Sharkey et al., 2007).

Total chlorophyll determination

The determination of total chlorophyll content and chlorophyll a/b ratios were performed according to Porra et al. (1989). 50-150 mg of fresh leaf material (3-12 cm^{-2}) from NT and *apx8* plants was first homogenized in 80% ice-cold acetone. The homogenate was centrifuged at 14,000 x g for 5 min. The supernatant obtained was used for spectrophotometric quantification of chlorophyll *a* and *b*. The absorbance was measured in two wavelengths, 663

and 646 nm (Porra et al., 1989). The chlorophyll content was expressed as μg chlorophyll cm^{-2}.

Ascorbate assays

Reduced ascorbate (ASC) and total ascorbate [ASC + oxidized ascorbate (DHA)] were assayed according to previously described (Kampfenkel et al., 1995). The assay of reduced ascorbate is based on the reduction of Fe^{3+} to Fe^{2+} by ASC and total ascorbate was measured after complete reduction of total ascorbate with 10 mM dithiothreitol (DTT). The excess DTT was removed by use of 0.5% (m/v) N-ethylmaleimide (NEM). The absorbance of Fe^{2+} complexed with 2,2′-bipirydyl was measured at 525 nm in a spectrophotometer. The DHA content was obtained by subtracting the reduced fraction from the total content. The ascorbate redox state was calculated as [(ASC)/(ASC + DHA)] x 100] and expressed as percentage. Ascorbate content was expressed as μmol ASC(DHA) g^{-1} fresh matter (FM).

Hydrogen peroxide (H_2O_2) content

Hydrogen peroxide content was measured using the Amplex®-red kit (Thermo Fisher Scientific®, USA), based on colorimetric measure of resorufin formation in presence of H_2O_2 (Zhou et al., 1997). Fresh leaf samples were macerated with liquid N_2 in presence of 100 mM K-phosphate buffer pH 7.5 and centrifuged at 12,000 x *g* (4 ºC) during 30 min. The supernatant was immediately used for H_2O_2 determination. The absorbance at 560 nm was quantified for H_2O_2 measurement. The hydrogen peroxide content was calculated from a standard curve, and the results were expressed as nmol H_2O_2 g^{-1} fresh mater (FM).

Protein extraction and APX activity assays

Fresh leaf samples were ground to a fine powder in the presence of liquid N_2 using a mortar and pestle and extracted on ice-cold (4 °C) and 100 mM K-phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 2 mM ascorbic acid (to prevent APX denaturation). The homogenate was centrifuged at 15,000x g for 30 min, and the obtained supernatant was used for determination of enzymatic activities. All steps were performed under low temperature (2-4 °C). The protein content was measured by Bradford's method (Bradford, 1976), utilizing BSA as a standard. APX activity was assayed in a reaction mixture containing 0.5 mM ascorbate and 0.1 mM EDTA dissolved in 100 mM K-phosphate buffer (pH 7.0) and enzyme extract. The reaction started by adding 3 mM H_2O_2. Enzyme activity was measured by following the decrease in absorbance at 290 nm (25 °C) over 180 s (Nakano and Asada, 1981). APX activity was calculated from the ASC molar extinction coefficient (2.8 mM^{-1} cm^{-1}) and activity was expressed as μmol ASC mg^{-1} protein min^{-1}. The APX activity was also assayed in presence of 30 μM pCMB, in order to correct the interference of class III peroxidases in APX activity.

Results

Thylakoidal APX knockdown generates impairment in shoot biomass and seed productivity in rice

In order to assess the importance of thylakoidal APX in rice photoprotective mechanisms, RNAi silenced plants were obtained. In total, five different plant lines were generated and evaluated regarding its phenotypical features (Supplementary material Fig. S1). Despite none of the transformed plants exhibited significant differences in shoot length in comparison to NT (Tukey test; $p \leq 0.05$), rice lines L2, L7 and L11 exhibited reduced shoot biomass accumulation in about 20% (Tukey test; $p \leq 0.05$), also compared to NT plants

(Supplementary material Fig. S1A-B). In addition, seed productivity and 100 seeds biomass was reduced by 30% (Tukey test; $p \leq 0.05$) only in rice lines L6 and L7, in comparison to NT (Supplementary material Fig. S1C-D). Therefore, since line L7 exhibited the most remarkable phenotypic effects, this line was selected for further experiments. This silenced rice line will be identified as *apx8* throughout the text. Figure 1 displays the morphological aspect of NT and rice *apx8* plants at approximately 45 days old. This figure corroborates the reduced biomass accumulation in silenced plants as compared to NT.

Silenced rice plants apx8 exhibited reduced light tolerance associated to impairment in NPQ formation

In order to investigate the photoprotective mechanisms that could be associated with thylakoidal APX in rice, chlorophyll *a* fluorescence parameters were evaluated in both NT and *apx8* plants grown under moderate light regime (400 μmol m^{-2} s^{-1}, 12h photoperiod). Silenced rice exhibited similar levels of Fv/Fm (Tukey test; $p \leq 0.05$) in comparison to NT plants, indicating that the maximum capacity of PSII reaction centers was not affected by thylakoidal APX reduced expression (Supplementary material Fig. S2). Since the dark condition to measure Fv/Fm parameters represents a disadvantageous rupture in light treatments, a new methodology to assess plant photoinhibition based on qPd parameter was employed (Ruban and Murchie, 2012). Silenced rice plants exhibited lower onset of photoinhibition ($qPd \leq 0.98$) in comparison to NT plants (Fig. 2; Tukey test; $p \leq 0.05$). Moreover, the minor qPd values reached in *apx8* plants were approximately 10% lower than the values exhibited by NT (Fig. 2; Tukey test; $p \leq 0.05$). Accordingly, figure 3 shows that *apx8* plants presented predominance of photoinhibited leaves (represented by greyscale fulfilled circles) at much lower light intensities as regarding NT (Fig. 3).

As evidenced previously, qPd based photoinhibition quantification is dependent on pNPQ formation levels (Ware et al., 2014; Carvalho et al., 2015; Giovagnetti and Ruban, 2015). Consequently, the relationship between light intensity and most efficient pNPQ in rice plants evidenced that NT plants exhibited a more efficient capability of pNPQ formation, demanding lower NPQ values to confer similar level of protection during increasing of light intensity, in comparison to *apx8* (Fig. 4A). These evidences suggest that NPQ formation process is somehow affected in thylakoidal APX deficient plants. Indeed, NPQ induction curves performed employing 400 and 1150 μmol m^{-1} s^{-1} of actinic light corroborated the existence of a delay in *apx8* NPQ formation, in comparison to NT plants (Fig. 4B). Especially employing 400 μmol m^{-1} s^{-1} of actinic light, *apx8* plants presented about 1 min of delay in fast NPQ induction, which is greatly associated with qE component (Fig. 4B). In addition, the dark relaxation of *apx8* rice plants after 400 μmol m^{-1} s^{-1} of actinic light is much less efficient than that exhibited by NT plants (Fig. 4B). Finally, experiments regarding the infiltration of NT leaf segments with 30 μM p-Chloromercuribenzoic acid (pCMB) corroborates that pharmacological reduction in APX activity could generate impairment in NPQ formation similar to 4 μM nigericin at moderate light and without any side effects on Fv/Fm (Supplementary material Fig. S3; Tukey test; $p \leq 0.05$).

NPQ impairment in apx8 knockdown plants are probably not related to ∆pH or violaxanthin deepoxidase activity

The Figure 5 suggests that *apx8* plants present no significant difference in the PSII electron transport rates in response to light intensity increases, as compared to NT plants (Fig. 5; Z-test; $p \leq 0.05$). This data suggests that probably the deficiency in thylakoidal APX and, therefore, pseudo-cyclic electron transport is not capable to explain NPQ formation impairment. In addition, leaf segments infiltration experiments employing 5 mM DTT (violaxanthin

deepoxidase inhibitor) and 4 μM nigericin (ΔpH formation inhibitor), evidenced that NPQ is similarly impaired in both NT and *apx8* plants in response to each respective pharmacological inhibitor (Fig. 6; Tukey test; $p \leq 0.05$). Finally, no significant differences in state-transition parameters associated to NPQ (qT and qS) were observed between NT and *apx8* rice plants (Supplementary material Table S1; Tukey test; $p \leq 0.05$).

Light dependent changes in NPQ dynamics are associated to modulation of H_2O_2 and chlorophyll content

In order to evaluate metabolic processes closely related to NPQ and oxidative stress that could have been affected by thylakoidal APX knockdown in rice, the ascorbate, H_2O_2 and total chlorophyll contents were assessed under both moderate and high light conditions, in the current study. Total ascorbate content, as well as ASC redox state did not present significant differences between NT and *apx8* plants under both light regimes studied (Fig. 7A; Tukey test; $p \leq 0.05$). In contrast, *apx8* plants exhibited approximately 20% higher H_2O_2 content in comparison to NT plants under moderate light conditions (Fig. 7B; Tukey test; $p \leq 0.05$). When these plants were exposed to high light for 4 days, NT plants exhibited a slight increase in H_2O_2 content (~10%), and *apx8* plants exhibited 19% decrease in this molecule levels (Fig. 7B; Tukey test; $p \leq 0.05$). In parallel, under moderate light conditions, *apx8* plants displayed about 40% less chlorophyll content in comparison to NT plants (Fig. 7C; Tukey test; $p \leq 0.05$). Nevertheless, under high light conditions, NT and *apx8* plants exhibited an induction of 130% and 260% in the chlorophyll levels, respectively, compared to moderate light conditions (Fig. 7C; Tukey test; $p \leq 0.05$). Therefore, under high light conditions, no significant differences between NT and *apx8* plants, in terms of chlorophyll content, was evident (Fig. 7C; Tukey test; $p \leq 0.05$).

In order to test the hypothesis that these changes in H_2O_2 and CHL content could be related to difference in NPQ formation between NT and *apx8*, new assays on NPQ induction

kinetics were performed. Employing the same actinic light (1000 µmol m^{-2} s^{-1}), *apx8* plants exhibited a delay in NPQ induction compared to NT, only when plants were acclimated to moderate light conditions (Fig. 7D; Tukey test; $p \leq 0.05$). When the plants were exposed to high light conditions, the differences in NPQ formation between the studied rice lines are less evident (Fig. 7E; Tukey test; $p \leq 0.05$). In addition, despite APX activity did not display significant differences between NT and *apx8* plants under moderate light, its activity was greatly induced (55%) in *apx8* plants under high light conditions (Fig. 8; Tukey test; $p \leq 0.05$). Higher APX activity in *apx8* plants under high light could have been responsible by the reduced H_2O_2 levels observed in these plants, in relation to NT.

Continuous moderate light is more harmful to apx8 rice plants than continuous high light

In order to evaluate the consequences of extended photoperiod in thylakoidal APX knockdown rice plants, NT and *apx8* plants were exposed to 24 hours of moderate light (400 µmol m^{-2} s^{-1}) and high light (2000 µmol m^{-2} s^{-1}). In the previous high light experiment (keeping 12 h photoperiod), *apx8* exhibited just slight reductions in terms of CO_2 assimilation (P_N-Ci curves), under both moderate and high light conditions (Supplementary material Fig. S4; Tukey test; $p \leq 0.05$). In contrast, after 24 hours of moderate light, *apx8* rice plants exhibited approximately 25% less CO_2 assimilation rates than NT plants (Fig. 9A; Tukey test; $p \leq 0.05$). Under continuous high light, no significant differences in P_N occurred between NT and *apx8* plants (Fig. 9A; Tukey test; $p \leq 0.05$). In parallel, these rice lines did not display significant differences in terms of stomatal conductance, Ci and transpiration under both continuous light regimes studied (Fig. 9B-D; Tukey test; $p \leq 0.05$). Interestingly, nonsignificant differences in CO_2 assimilation between NT and *apx8* plants were observed until 12 hours of continuous moderate light (Supplementary material Fig. S3; Tukey test; $p \leq 0.05$). Nevertheless, in the presence of 125 µM methyl viologen, *apx8* exhibited a greater impairment in CO_2 assimilation

than NT plants after 12 hours of light treatment (Supplementary material Fig. S5; Tukey test; $p \leq 0.05$), which is an additional indicative that photosynthesis limitations observed in *apx8* plants is related to oxidative unbalance.

Figure 10A displays hydrogen peroxide content in NT and *apx8* rice plants exposed to continuous moderate and high light. Under continuous moderate light, *apx8* plants display a slight decrease in H_2O_2 (Tukey test; $p \leq 0.05$). Under continuous high light, plant lines did not display significant differences (Tukey test; $p \leq 0.05$). Interestingly, under moderate continuous light *apx8* exhibited higher NPQ formation in comparison to NT plants, but, similarly to H_2O_2 content, no significant differences were observed among plant lines under continuous high light (Fig. 10B, Tukey test; $p \leq 0.05$). In parallel, *apx8* plants exhibited lower ascorbate reduced state in comparison to NT plants under continuous moderate light. In opposition and similarly to the pattern observed for H_2O_2 and NPQ, the two rice lines did not display significant ascorbate reduced-state differences in response to continuous high light. (Fig. 10B; Tukey test; $p \leq 0.05$).

Discussion

In the current study, *apx8* plants exhibited higher H_2O_2 levels in comparison to NT plants, under ML. Chloroplast ascorbate peroxidases are responsible for 1/5 of total APX activity in plant cells (Amako et al., 1994). In addition, H_2O_2 production rate in chloroplasts of mesophyll cells during photosynthesis is estimated in 4 $\mu mol\ m^{-2}\ s^{-1}$ (Foyer and Noctor, 2003). Over-accumulation of H_2O_2 in chloroplasts is thought to generate a specific inactivation at the elongation factor G by intramolecular disulphide bond (Nishiyama et al., 2011). The inactivation of this protein affects the *de novo* protein synthesis, consequently leading to delay in D1 protein synthesis, affecting the PSII reaction centers (RCII) repair mechanism and generating photoinhibition (Nishiyama et al., 2011). However, as evidenced by our data the

levels of this ROS probably were not high enough to generate disturbances in the RC repair mechanisms, since both rice lines exhibited similar Fv/Fm under moderate light conditions.

A. thaliana plants knockout (KO) for thylakoidal APX (*tapx*), similarly to rice, did not exhibited contrasting Fv/Fm in comparison to wild type under moderate light growth conditions (Giacomelli et al., 2007; Kangasjärvi et al., 2008; Maruta et al., 2010). In addition, *Arabidopsis tapx* plants do not exhibit phenotypic differences in comparison to wild type, under moderate light conditions (Giacomelli et al., 2007; Kangasjärvi et al., 2008; Maruta et al., 2010). Interestingly, the phenotypic effects of tAPX silencing in rice plants is very distinct from the reported for KO-*Arabidopsis*. Our data evidenced that *apx8* rice plants display approximately 20% less shoot biomass and seed productivity as compared to NT. Taken together, these data indicate that thylakoidal APX importance in plants seems to be species-dependent.

Why rice plants deficient in thylakoidal APX expression exhibit different phenotypic traits in comparison to NT, despite apparently no side effects in maximum quantum efficiency of PSII? P_N-Ci curves suggest that CO_2 assimilation is just slightly decreased in *apx8*, compared to NT plants (Fig. S4). This effect certainly has contributed in some extension to the *apx8* rice phenotype. However, thylakoid APX are closely related to photosynthetic electron transport chain (Asada, 1999; Maruta et al., 2016) and, therefore, this apparent miss-connection is very intriguing. Could Fv/Fm approach not be suitable enough to assess the importance of thylakoidal APX in rice plants? In order to answer this question, a new fluorescence-based methodology for the photoinhibition onset quantification, based on the coefficient of photochemical quenching measured in the dark following illumination (qPd), as reported recently by Ruban and Murchie (2012), presents a great potential for comparative plant light tolerance studies (Carvalho et al., 2015; Giovagnetti and Ruban, 2015; Ware et al., 2015).

Employing this new methodology, *apx8* plants exhibited 50% of leaves already photoinhibited under light intensities approximately 60% lower than NT plants. Moreover, silenced rice plants reached qPd values significantly lower than NT, after a sequence of

increased light exposure (0-2000 μmol m^{-2} s^{-1}). Previous data have evidenced that in *Arabidopsis*, qPd stability is strongly dependent on NPQ (Ware et al., 2014) and is also associated with oxygen evolving activity (Giovagnetti and Ruban, 2015). The use of qPd-related methodologies also allowed the quantification of exact fraction of NPQ that is related to photoprotection, pNPQ. Accordingly, pNPQ in *apx8* plants was less efficient than in NT, which strongly suggests that *apx8* plants should probably display deficiency in terms of NPQ mechanisms.

The NPQ induction kinetics assays performed in the current study clearly corroborate that *apx8* plants exhibit a delay in NPQ formation. The idea that thylakoidal APX could be associated with NPQ protection is not new in the literature. During 1990's years, few works have reported that the pharmacological inhibition of APX activity using KCN is related to impairment in NPQ formation (Neubauer and Yamamoto, 1992). It has been proposed that the Mehler-peroxidase reaction favours the linear electron transport in thylakoid membranes (Neubauer and Yamamoto, 1992). This electron flux, also referred as water-water cycle (Asada, 1999), should induce ∆pH formation and, therefore inducing NPQ (Neubauer and Yamamoto, 1992; Hormann et al., 1994; Neubauer and Yamamoto, 1994).

However, as evidenced in the current study by nigericin infiltration, *apx8* plants apparently do not present limitation in terms of ∆pH formation. Moreover, since NT and *apx8* exhibited PSII electron transport rates very close, it is less probable that NPQ (especially qE) differences are related to ∆pH. In other hand, violaxanthin-deepoxidase activity is another crucial event for qE generation, which, as APX, is also dependent on ascorbate as substrate (Demmig-Adams et al., 1996; Ruban et al., 2003). Our data suggest that violaxanthin-deepoxidase route was not affected by the thylakoidal APX knockdown in rice: 1) Ascorbate content are similar in NT and *apx8* plants under moderate light conditions and; 2) NT and *apx8* rice plants are similarly affected by DTT inhibition in terms of NPQ formation. In other hand, PsbS proteins that possibly play a crucial role in sensing lumen acidification and transducing

the signal to the antenna to induce qE (Funk et al., 1995; Li et al., 2000; Niyogi et al., 2005), were not quantified in the current study.

Thus, an important question remains open: which mechanism could be responsible by the differences exhibited by *apx8* rice plants in terms of NPQ formation? In the current study, two important parameters were significantly changed in *apx8* plants: H_2O_2 and chlorophyll content. Recent studies have reported that higher H_2O_2 in *Arabidopsis* plants under high light acclimation is related to negative modulation of *lhcb* genes expression (Li et al., 2009; Borisova-Mubarakshina et al., 2015). These genes encode for important light harvest antenna proteins and the most recent hypothesis support that qE site is related to photosynthetic pigments, including chlorophylls, associated to PSII antennas (Chmeliov et al., 2015; Ruban, 2016).

Chlorophyll molecules display several roles in antennae function and composition, which have been known for a long time, contributing to both the capture and dissipation of energy (Peter and Thornber, 1991; Bassi and Dainese, 1992; Ruban et al., 1999; Hogewoning et al., 2012; van Amerongen and Croce, 2013). Moreover, recently it has been reported that pNPQ parameter is well correlated with leaf chlorophyll content variations throughout *Arabidopsis* ontogenesis and associated with the onset of photoinhibition (Carvalho et al., 2015). Indeed, photoinhibition of PSII can occur as consequence of the produced singlet oxygen as well as by weakly coupled chlorophyll molecules (Goh et al., 2012). Thus, increased H_2O_2 and reduced chl content in *apx8* leaves might have importance for NPQ impairment in these plants.

Interestingly, when plants were acclimated for 4 days at high light conditions, the levels of H_2O_2 in *apx8* rice was lower than that exhibited by NT. These results were accompanied to similar chl contents and similar NPQ induction kinetics in both plant lines. Taken together, these finds reinforce the possible involvement of H_2O_2 and chl levels in the lower NPQ formation exhibited by *apx8* plants. However, an interesting question, is why H_2O_2 content was

not accumulated in *apx8* plants acclimated to high light conditions? Indeed, it is well known that a great fraction of the H_2O_2 produced in chloroplasts is able to cross its membranes and reach other cellular compartments, such as cytosol. Therefore, the great importance of other APX isoforms, especially in cytosol, acting in a compensatory response must be relevant (Mubarakshina et al., 2010; Bonifacio et al., 2011). Accordingly, high light acclimated *apx8* plants exhibited higher total APX activity than NT. Moreover, in tAPX-KO *Arabidopsis* plants acclimated to high light, it has been reported that 2-cys-peroxiredoxin proteins are up-regulated (Kangasjärvi et al., 2008). Further studies could clarify if a similar mechanism was triggered in *apx8* rice plants acclimated to high light conditions.

Despite of *apx8* rice plants exhibit restricted shoot biomass and seed productivity, accompanied by lower photoinhibition onset associated to the lower pNPQ, this silenced rice is able to effective acclimation to high light (Giacomelli et al., 2007; Kangasjärvi et al., 2008; Maruta et al., 2010). However, under continuous moderate light (24 h) *apx8* exhibited higher impairment in photosynthesis and ascorbate oxidation than NT. The complete interpretation of such data is complex and demands further investigation. However, it is possible to speculate that, as usually discussed in literature, each specific stress condition (in this case, light intensity) is related to a specific signalling event, which in turns could activate a singular set of responses (Karpiński et al., 2013; Suzuki et al., 2013; Gilroy et al., 2016). This issue has also been amply discussed by our group in terms of rice plants silenced in other APX isoforms (Rosa et al., 2010; Bonifacio et al., 2011; Ribeiro et al., 2012; Carvalho et al., 2014; Caverzan et al., 2014; Sousa et al., 2015).

Taken together, our data clearly suggest that thylakoidal APX silencing in rice is involved with shoot biomass and seed productivity reduction, which was associated to lower onset of photoinhibition under increase of light intensity. These features were accompanied by lower NPQ induction, which probably was associated to lower chlorophyll levels and higher H_2O_2 contents in the silenced rice leaves. Surprisingly, despite these deleterious features, *apx8*

plants are capable to acclimate to high light conditions, probably due to recovery of APX activity and control of H_2O_2 levels. Nevertheless, further studies are still needed in order to understand completely the physiological roles of thylakoidal APX in plants.

Acknowledgements

This work was supported by National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico CNPq) – Proc. 486231-2012-7.

References

Amako K, Chen G-XG, Asada K (1994) Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant Cell Physiol **35**: 497–504

van Amerongen H, Croce R (2013) Light harvesting in photosystem II. Photosynth Res **116**: 251–263

Asada K (1999) The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol **50**: 601–639

Bassi R, Dainese P (1992) A supramolecular light-harvesting complex from chloroplast photosystem-II membranes. Eur J Biochem **204**: 317–326

Bechtold U, Richard O, Zamboni A, Gapper C, Geisler M, Pogson B, Karpinski S, Mullineaux PM (2008) Impact of chloroplastic- and extracellular-sourced ROS on high light-responsive gene expression in Arabidopsis. J Exp Bot **59**: 121–133

Bonifacio A, Martins MO, Ribeiro CW, Fontenele AV, Carvalho FEL, Margis-Pinheiro M, Silveira JAG (2011) Role of peroxidases in the compensation of cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress. Plant, Cell Environ **34**: 1705–1722

Borisova-Mubarakshina MM, Ivanov BN, Vetoshkina DV, Lubimov VY, Fedorchuk TP, Naydov IA, Kozuleva MA, Rudenko NN, Dall'Osto L, Cazzaniga S, et al (2015) Long-term acclimatory response to excess excitation energy: evidence for a role of hydrogen peroxide in the regulation of photosystem II antenna size. J Exp Bot **66**: 7151–7164

Carvalho FEL, Ribeiro CW, Martins MO, Bonifacio A, Staats CC, Andrade CMB, Cerqueira JV, Margis-Pinheiro M, Silveira JAG (2014) Cytosolic APX knockdown rice plants sustain photosynthesis by regulation of protein expression related to photochemistry, Calvin cycle and photorespiration. Physiol Plant **150**: 632–645

Carvalho FEL, Ware MA, Ruban AV (2015) Quantifying the dynamics of light tolerance in *Arabidopsis* plants during ontogenesis. Plant Cell Environ **38**: 2603–2617

Caverzan A, Bonifacio A, Carvalho FEL, Andrade CMB, Passaia G, Schünemann M, Maraschin F dos S, Martins MO, Teixeira FK, Rauber R, et al (2014) The knockdown of chloroplastic ascorbate peroxidases reveals its regulatory role in the photosynthesis and protection under photo-oxidative stress in rice. Plant Sci **214**: 74–87

Chmeliov J, Bricker WP, Lo C, Jouin E, Valkūnas L, Ruban A, Duffy CDP (2015) An "all pigment" model of excitation quenching in LHCII. Phys Chem Chem Phys **17**: 15857–15867

Davletova S, Rizhsky L, Liang H, Shengqiang Z, Oliver DJ, Coutu J, Shulaev V, Schlauch K, Mittler R (2005) Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis. Plant Cell **17**: 268–281

Demmig-Adams B, Adams WW (1992) Photoprotection and other responses of plants to high light stress. Annu Rev Plant Physiol Plant Mol Biol **43**: 599–626

Demmig-Adams B, Adams WW, Planta IG (1996) Xanthophyll cycle and light stress in nature: uniform response to excess direct sunlight among higher plant species. Planta **198**: 460–470

Flexas J, Ribas-Carbó M, Diaz-Espejo A, Galmés J, Medrano H (2008) Mesophyll conductance to CO_2: Current knowledge and future prospects. Plant, Cell Environ **31**: 602–621

Foyer CH, Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant **119**: 355–364

Funk C, Schröder WP, Napiwotzki A, Tjus SE, Renger G, Andersson B (1995) The PSII-S protein of higher plants: A new type of pigment- binding protein. Biochemistry **34**: 11133–11141

Giacomelli L, Masi A, Ripoll DR, Lee MJ, Van Wijk KJ (2007) *Arabidopsis thaliana*

deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low. Plant Mol Biol **65**: 627–644

Gilroy S, Białasek M, Suzuki N, Górecka M, Devireddy Amith R, Karpiński S, Mittler R (2016) ROS, calcium and electric signals: key mediators of rapid systemic signaling in plants. Plant Physiol ra45

Giovagnetti V, Ruban AV (2015) Discerning the effects of photoinhibition and photoprotection on the rate of oxygen evolution in Arabidopsis leaves. J Photochem Photobiol B Biol **152**: 272-278

Goh CH, Ko SM, Koh S, Kim YJ, Bae HJ (2012) Photosynthesis and environments: photoinhibition and repair mechanisms in plants. J Plant Biol **55**: 93–101

Hogewoning SW, Wientjes E, Douwstra P, Trouwborst G, van Ieperen W, Croce R, Harbinson J (2012) Photosynthetic quantum yield dynamics: from photosystems to leaves. Plant Cell **24**: 1921–1935

Hormann H, Neubauer C, Schreiber U (1994) An active Mehler-peroxidase reaction sequence can prevent cyclic PSI electron transport in the presence of dioxygen in intact spinach chloroplasts. Photosynth Res **41**: 429–437

Kampfenkel K, Van Montagu M, Inzé D, Vanmontagu M, Inzé D (1995) Extraction and determination of ascorbate and dehydroascorbate from plant tissue. Anal Biochem **225**: 165–167

Kangasjärvi S, Lepistö A, Hännikäinen K, Piippo M, Luomala E-M, Aro E-M, Rintamäki E (2008) Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses. Biochem J **412**: 275–285

Karpiński S, Szechyńska-Hebda M, Wituszyńska W, Burdiak P (2013) Light acclimation, retrograde signalling, cell death and immune defences in plants. Plant Cell Environ **36**: 736–744

Koussevitzky S, Suzuki N, Huntington S, Armijo L, Sha W, Cortes D, Shulaev V, Mittler R (2008) Ascorbate peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. J Biol Chem **283**: 34197–34203

Li XP, Björkman O, Shih C, Grossman AR, Rosenquist M, Jansson S, Niyogi KK (2000) A pigment-binding protein essential for regulation of photosynthetic light harvesting. Nature **403**: 391–395

Li Z, Wakao S, Fischer BB, Niyogi KK (2009) Sensing and responding to excess light. Annu Rev Plant Biol **60**: 239–260

Mano J, Ohno C, Domae Y, Asada K (2001) Chloroplastic ascorbate peroxidase is the primary target of methylviologen-induced photooxidative stress in spinach leaves: Its

relevance to monodehydroascorbate radical detected with in vivo ESR. Biochim Biophys Acta - Bioenerg **1504**: 275–287

Maruta T, Inoue T, Noshi M, Tamoi M, Yabuta Y, Yoshimura K, Ishikawa T, Shigeoka S (2012) Cytosolic ascorbate peroxidase 1 protects organelles against oxidative stress by wounding- and jasmonate-induced H_2O_2 in Arabidopsis plants. Biochim Biophys Acta **1820**: 1901–1907

Maruta T, Sawa Y, Shigeoka S, Ishikawa T (2016) Diversity and evolution of ascorbate peroxidase functions in chloroplasts: more than just a classical antioxidant enzyme? Plant Cell Physiol **0**: pcv203

Maruta T, Tanouchi A, Tamoi M, Yabuta Y, Yoshimura K, Ishikawa T, Shigeoka S (2010) Arabidopsis chloroplastic ascorbate peroxidase isoenzymes play a dual role in photoprotection and gene regulation under photooxidative stress. Plant Cell Physiol **51**: 190–200

Miki D, Shimamoto K (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. Plant Cell Physiol **45**: 490–495

Mubarakshina MM, Ivanov BN, Naydov IA, Hillier W, Badger MR, Krieger-Liszkay A (2010) Production and diffusion of chloroplastic H_2O_2 and its implication to signalling. J Exp Bot **61**: 3577–3587

Murata N, Allakhverdiev SI, Nishiyama Y (2012) The mechanism of photoinhibition in vivo: Re-evaluation of the roles of catalase, α-tocopherol, non-photochemical quenching, and electron transport. Biochim Biophys Acta **1817**: 1127–33

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbato specific peroxidase in spinach chloroplasts. Plant Cell Physiol **22**: 867–880

Neubauer C, Yamamoto HY (1992) Mehler-peroxidase reaction mediates zeaxanthin formation and zeaxanthin-related fluorescence quenching in intact chloroplasts. Plant Physiol **99**: 1354–1361

Neubauer C, Yamamoto HY (1994) Membrane barriers and Mehler-peroxidase reaction limit the ascorbate available for violaxanthin de-epoxidase activity in intact chloroplasts. Photosynth Res **39**: 137–147

Nishiyama Y, Allakhverdiev SI, Murata N (2011) Protein synthesis is the primary target of reactive oxygen species in the photoinhibition of photosystem II. Physiol Plant **142**: 35–46

Niyogi KK, Li X-P, Rosenberg V, Jung H-S (2005) Is PsbS the site of non-photochemical quenching in photosynthesis? J Exp Bot **56**: 375–82

Ogawa K, Kanematsu S, Asada K (1997) Generation of superoxide anion and localization

of CuZn-superoxide dismutase in the vascular tissue of spinach hypocotyls: their association with lignification. Plant Cell Physiol **38**: 1118–1126

Peter GF, Thornber JP (1991) Biochemical composition and organization of higher plant photosystem II light -harvesting pigment-proteins. J Biol Chem **266**: 16745–16754

Pilon M, Ravet K, Tapken W (2011) The biogenesis and physiological function of chloroplast superoxide dismutases. Biochim Biophys Acta **1807**: 989–998

Pnueli L, Liang H, Rozenberg M, Mittler R (2003) Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (Apx1)-deficient Arabidopsis plants. Plant J **34**: 187–203

Porra RJR, Thompson WWA, Kriedemann PPE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: Biochim Biophys Acta **975**: 384–394

Ribeiro CW, Carvalho FEL, Rosa SB, Alves-Ferreira M, Andrade CMB, Ribeiro-Alves M, Silveira JAG, Margis R, Margis-Pinheiro M (2012) Modulation of genes related to specific metabolic pathways in response to cytosolic ascorbate peroxidase knockdown in rice plants. Plant Biol **14**: 944–955

Rosa SB, Caverzan A, Teixeira FK, Lazzarotto F, Silveira JAG, Ferreira-Silva SL, Abreu-Neto J, Margis R, Margis-Pinheiro M (2010) Cytosolic APx knockdown indicates an ambiguous redox responses in rice. Phytochemistry **71**: 548–558

Ruban AV (2016) Non-photochemical chlorophyll fluorescence quenching: mechanism and effectiveness in protection against photodamage. Plant Physiol **70**: 1903–1916

Ruban AV, Belgio E (2014) The relationship between maximum tolerated light intensity and photoprotective energy dissipation in the photosynthetic antenna: chloroplast gains and losses. Philos Trans R Soc B Biol Sci **369**: 20130222–20130222

Ruban AV, Murchie EH (2012) Assessing the photoprotective effectiveness of non-photochemical chlorophyll fluorescence quenching: A new approach. Biochim Biophys Acta - Bioenerg **1817**: 977–982

Ruban AV, Rees D, Pascal AA, Horton P (1992) Mechanism of ΔpH-dependent dissipation of absorbed excitation energy by photosynthetic membranes. II. The relationship between LHCII aggregation in vitro and qE in isolated thylakoids. Biochim Biophys Acta - Bioenerg **1102**: 39–44

Ruban AV, Lee PJ, Wentworth M, Young AJ, Horton P (1999) Determination of the stoichiometry and strength of binding of xanthophylls to the photosystem II light harvesting complexes. J Biol Chem **274**: 10458–10465

Ruban AV, Wentworth M, Yakushevska AE, Andersson J, Lee PJ, Keegstra W,

Dekker JP, Boekema EJ, Jansson S, Horton P (2003) Plants lacking the main light-harvesting complex retain photosystem II macro-organization. Nature **421**: 648–652

Sewelam N, Jaspert N, Van Der Kelen K, Tognetti VB, Schmitz J, Frerigmann H, Stahl E, Zeier J, Van Breusegem F, Maurino VG (2014) Spatial H_2O_2 signaling specificity: H_2O_2 from chloroplasts and peroxisomes modulates the plant transcriptome differentially. Mol Plant **7**: 1191–1210

Sharkey TD, Bernacchi CJ, Farquhar GD, Singsaas EL (2007) Fitting photosynthetic carbon dioxide response curves for C3 leaves. Plant, Cell Environ **30**: 1035–1040

Shigeoka S, Maruta T (2014) Cellular redox regulation, signaling, and stress response in plants. Biosci Biotechnol Biochem **78**: 1457–1470

Sousa RHV, Carvalho FEL, Ribeiro CW, Passaia G, Cunha JR, Lima-Melo Y, Margis-Pinheiro M, Silveira JAG (2015) Peroxisomal APX knockdown triggers antioxidant mechanisms favourable for coping with high photorespiratory H_2O_2 induced by CAT deficiency in rice. Plant Cell Environ **38**: 499–513

Suzuki N, Miller G, Salazar C, Mondal HA, Shulaev E, Cortes DF, Shuman JL, Luo X, Shah J, Schlauch K, et al (2013) Temporal-spatial interaction between reactive oxygen species and abscisic acid regulates rapid systemic acclimation in plants. Plant Cell **25**: 3553–3569

Teixeira FK, Menezes-Benavente L, Galvão VC, Margis R, Margis-Pinheiro M (2006) Rice ascorbate peroxidase gene family encodes functionally diverse isoforms localized in different subcellular compartments. Planta **224**: 300–314

Teixeira FK, Menezes-Benavente L, Margis R, Margis-Pinheiro M (2004) Analysis of the molecular evolutionary history of the ascorbate peroxidase gene family: Inferences from the rice genome. J Mol Evol **59**: 761–770

Upadhyaya NM, Zhou X-R, Zhu Q-H, Eamens A, Wang M-B, Water-House PM, Dennis ES (2000) Transgenic Rice. *In* L O'Brien, RJ Henry, eds, Transgenic Cereal., AACC. Minnesota, pp 28–87

Ware MA, Giovagnetti V, Belgio E, Ruban AV (2015) PsbS protein modulates non-photochemical chlorophyll fluorescence quenching in membranes depleted of photosystems. J Photochem Photobiol B Biol **152**: 301–307

Ware MA, Belgio E, Ruban AV (2014) Comparison of the protective effectiveness of NPQ in Arabidopsis plants deficient in PsbS protein and zeaxanthin. J Exp Bot **66**: 1259–1270

Yamaguchi K, Mori H, Nishimura M (1995) A novel isoenzyme of ascorbate peroxidase localized on glyoxysomal and leaf peroxisomal membranes in pumpkin. Plant Cell

Physiol **36**: 1157–1162

Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP (1997) A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. Anal Biochem **253**: 162–168

Zulfugarov IS, Tovuu A, Eu Y-J, Dogsom B, Poudyal RS, Nath K, Hall M, Banerjee M, Yoon UC, Moon Y-H, et al (2014) Production of superoxide from Photosystem II in a rice (*Oryza sativa* L.) mutant lacking PsbS. BMC Plant Biol **14**: 242

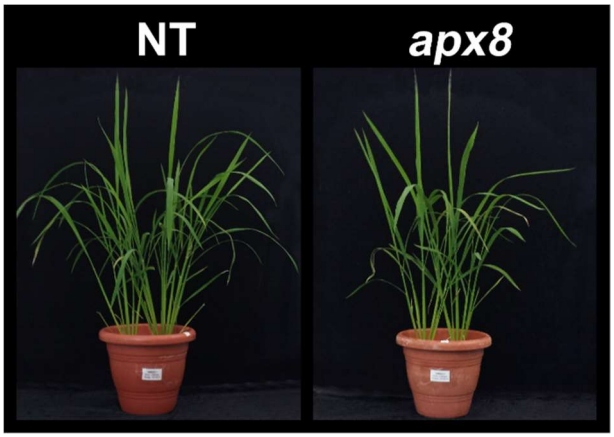

Figure 1. Phenotypic characterization of *apx8* silenced rice. Plants were cultivated under greenhouse conditions (30±5 °C, 50±20 % humidity, maximum PPFD average of 850 μmol^{-2} s^{-1} at noon and 12 hours of photoperiod) for 45 days. Figures are the most representative of 8 independent pots.

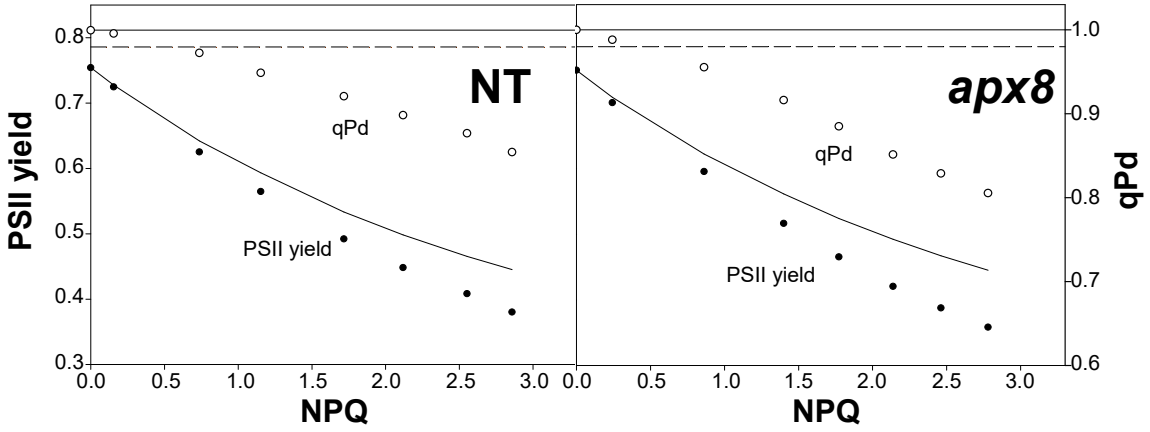

Figure 2. Relationship between PSII yield (closed circles), qPd (open circles) and NPQ parameters measured on intact leaves from measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Continuous line represents the theoretical yield, calculated according to Carvalho et al., (2015). Upper dashed lines represent the earliest symptom of photoinhibition, characterized by qPd values equal to 0.98. Error bars show the S.E. (n=24). Plants were acclimated to 400 µE m^{-2} s^{-1} and 12 h photoperiod.

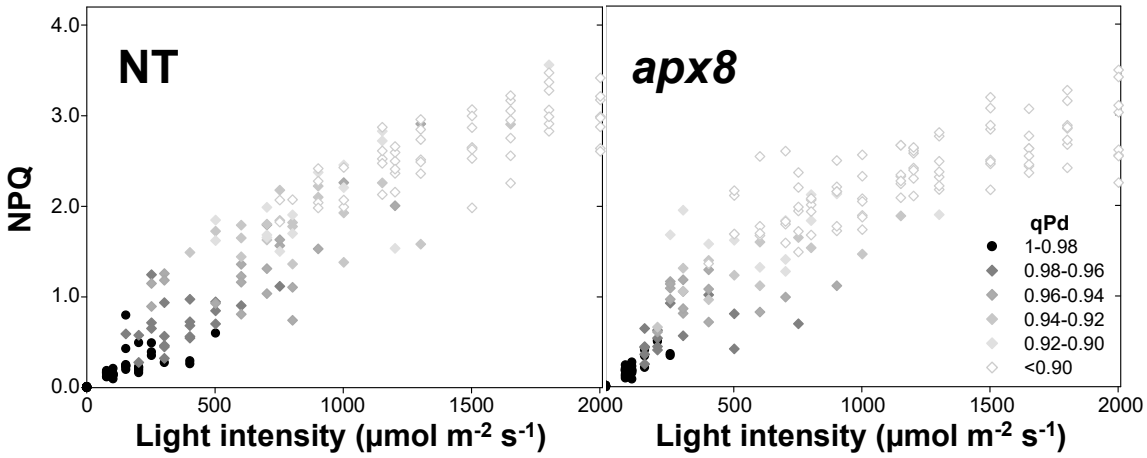

Figure 3. Relationship between NPQ, actinic light intensities and qP measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants were acclimated to 400 µE m^{-2} s^{-1} and 12 h photoperiod. Each circle represent a specific NPQ measure obtained at each light intensity. NPQ circles are represented in grayscale according to the correspondent qPd values.

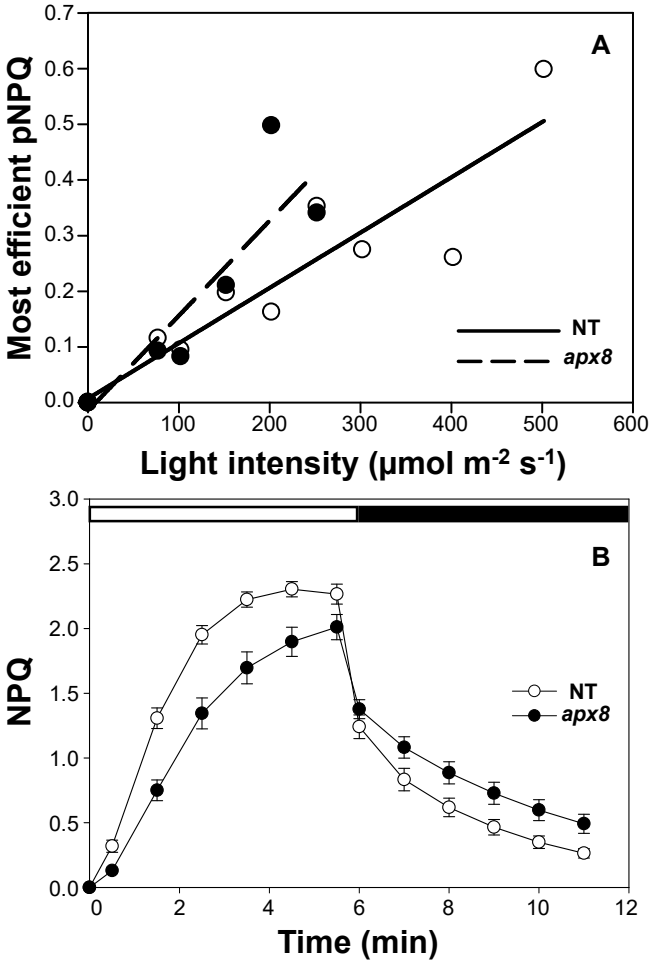

Figure 4. (A) Relationship between the most efficient pNPQ value and light intensity and (B) non-photochemical quenching measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants were acclimated to 400 µE m^{-2} s^{-1} and 12 h photoperiod. The lines represent regression fit curves (Polynomial; Linear, f = y0+a*x). The actinic light employed in NPQ induction kinetics was 400 µmol m^{-2} s^{-1}. The routine consisted of 5 min illumination (white rectangle) followed by 5 min dark exposure relaxing (dark rectangle). NPQ values are average of 8 independent measures on different leaves (n=8). Error bars represent standard error (S.E.).

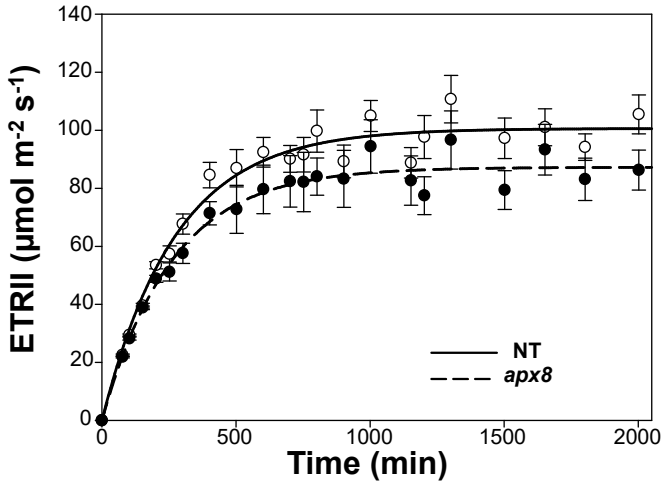

Figure 5. Photosystem II electron transport rate (ETRII) measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants were acclimated to 400 µE m^{-2} s^{-1} and 12 h photoperiod. ETRII values are average of 8 independent measures on different leaves (n=8). Error bars represent standard error (S.E). Continuous lines represent the exponential rise to maximum fitted curve, single-2 parameters [$y= a(1-e^{-bx})$], plotted using SigmaPlot12 (Systat Software, Inc., Chicago, IL, USA).

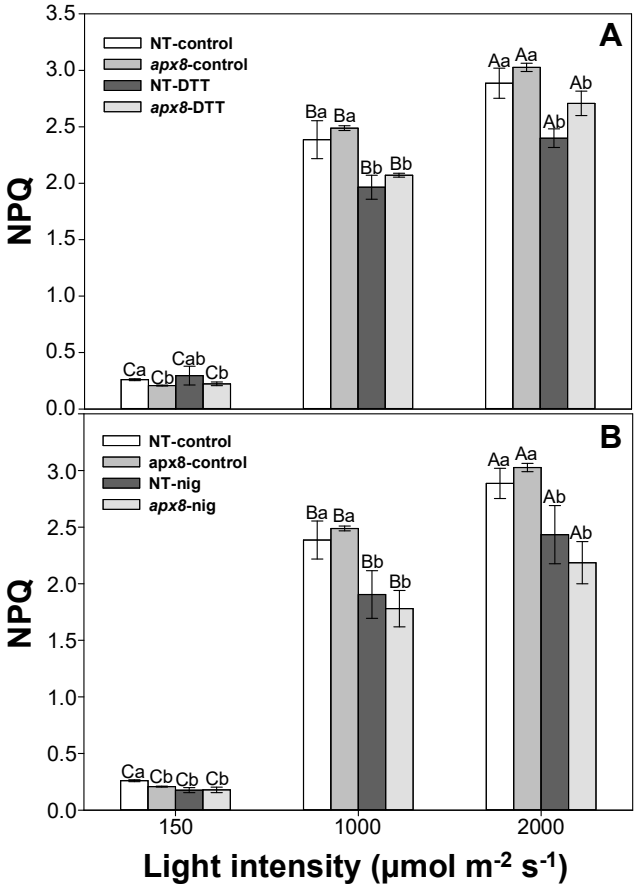

Figure 6. Non-photochemical quenching (NPQ) measured in leaf segments from non-transformed (NT) and thylakoidal APX silenced (*apx8*) rice. Plants grown at 400 µE m-2 s-1 and 12 h photoperiod. Leaf segments were infiltrated with 4 µM nigericin (NIG) or 5 mM dithiothreitol (DTT), inhibitors of delta pH and violaxanthin de-epoxidase activity, respectively. The values represent average of 3 independent assays on performed different leaves (n=3). Error bars represent standard error (S.E.). Different capital letters mean significant differences between light regimes and different lowercase letters represent significant differences between chemical treatments, according to Tukey's test, $p \leq 0.05$.

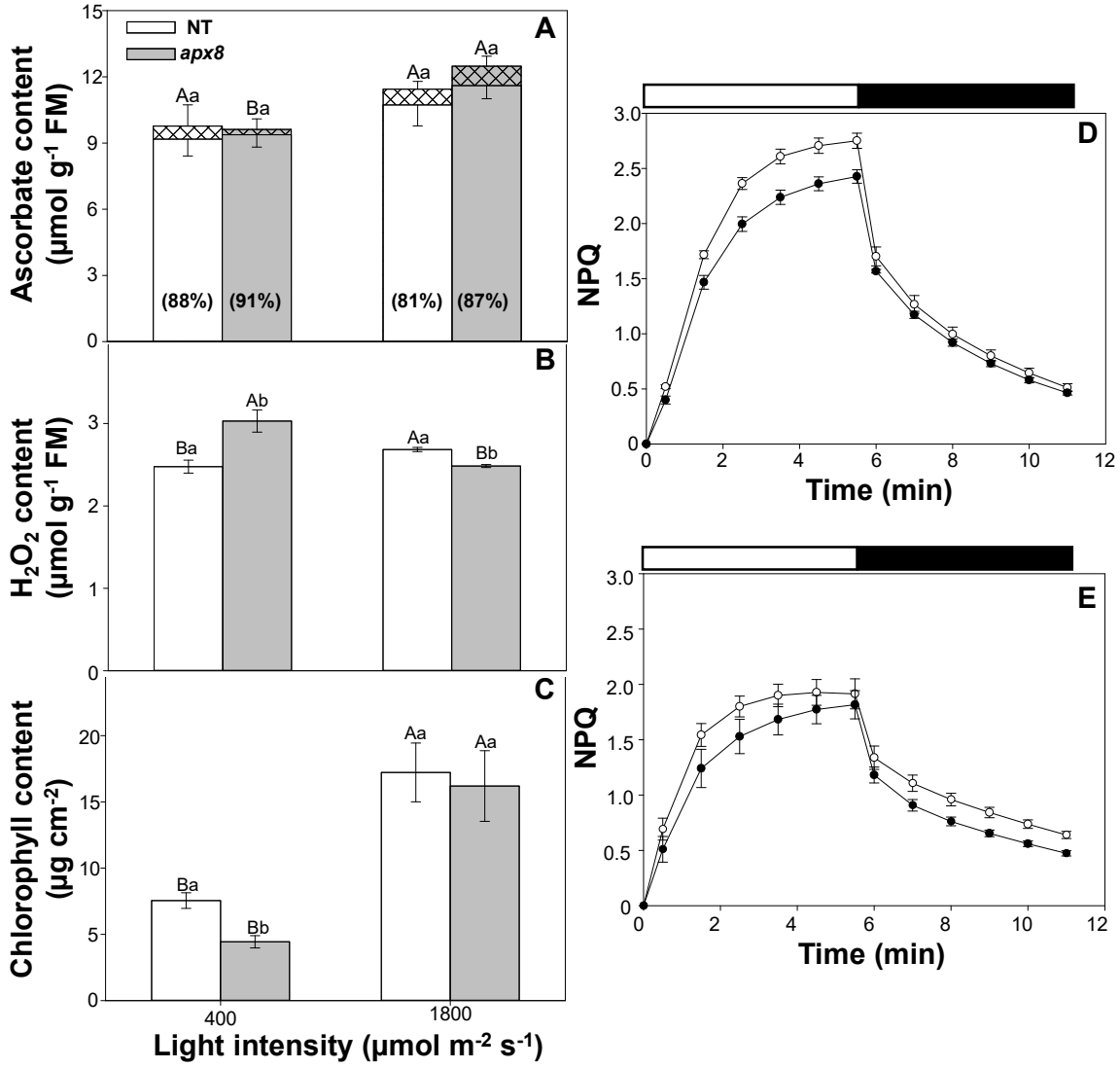

Figure 7. Redox indicators, chlorophyll content and NPQ in NT and apx8 plants exposed to high light. (A) Acorbate (ASC) and dehydroascorbate (DHA) content, (B) hydrogen peroxide levels and (C) total chlorophyll measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants grown at 400 µE m^{-2} s^{-1} and 12 h photoperiod and then transferred to 1,800 µE m^{-2} s^{-1} for 4 days. (D) NPQ induction kinetics measured in 400 µmol m^{-2} s^{-1} acclimated plants and (E) NPQ induction kinetics measured in 1800 µmol m^{-2} s^{-1} acclimated plants. ASC and DHA values are expressed in µmol per gram of fresh matter (g^{-1} F.M.) and are average of 3 independent measures on extracts from different leaves (n=3). (H_2O_2) values are expressed in µmol per gram of fresh matter (g^{-1} F.M.) and are average of 3 independent measures on extracts from different leaves (n=3). CHL values are expressed in µg per square centimetre (µg cm^{-2}) and are average of 3 independent measures on extracts from different leaves (n=3). Error bars represent standard error (S.E.). Different capital letters mean significant differences between light regimes and different lowercase letters represent significant differences between plant lines. The actinic light employed in NPQ induction kinetics was 1000 µmol m^{-2} s^{-1}. The routine consisted of 5 min illumination (white rectangle) followed by 5 min dark exposure relaxing (dark rectangle). NPQ values are average of 8 independent measures on different leaves (n=8). Error bars represent standard error (S.E.).

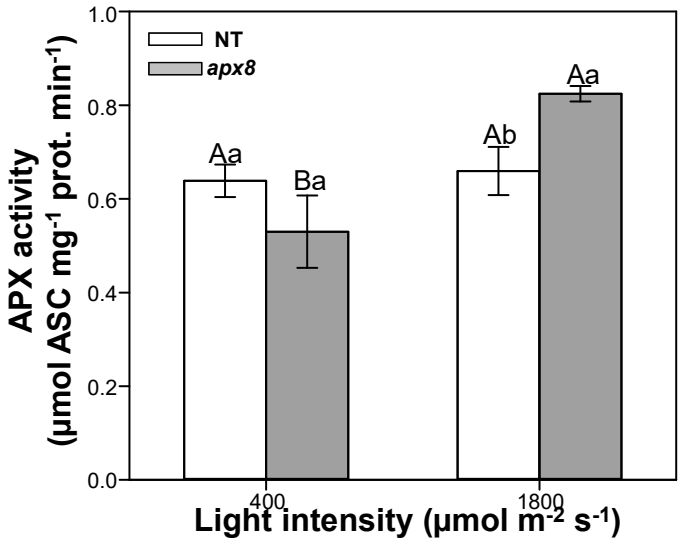

Figure 8. Total APX activity measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants grown at 400 µE m^{-2} s^{-1} and 12 h photoperiod were transferred to 1,800 µE m^{-2} s^{-1} for 4 days. APX activity is expressed in µmol ASC per milligram of protein (mg^{-1} protein) per minute and are average of 3 independent measures on extracts from different leaves (n=3). Error bars represent standard error (S.E.). Different capital letters mean significant differences between light regimes and different lowercase letters represent significant differences between plant lines, according to Tukey's test, $p \leq 0.05$.

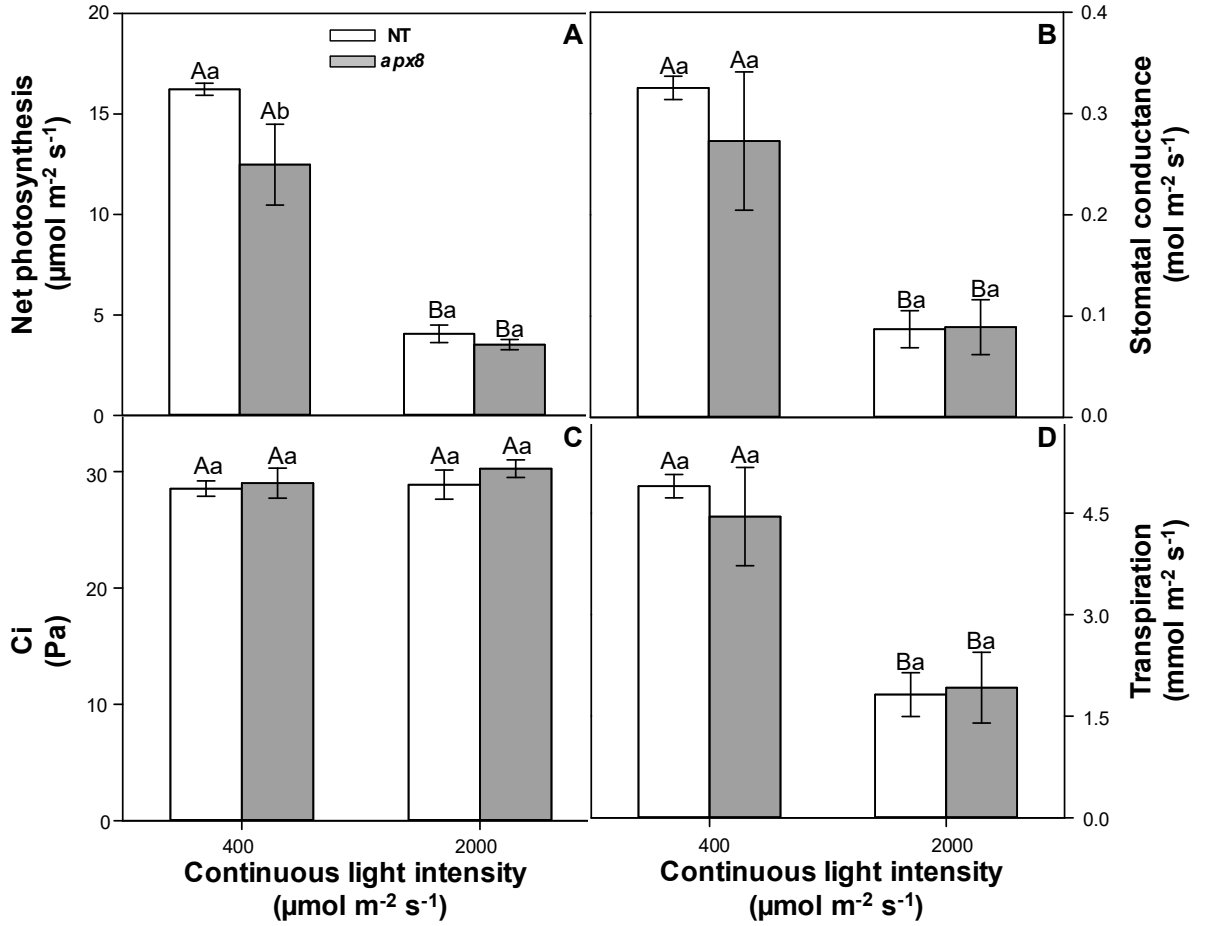

Figure 9. (A) Photosynthetic CO_2 assimilation (P_N), (B) stomatal conductance (g_S), (C) intracellular CO_2 concentration (Ci) and leaf transpiration (E) measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants grown at 400 µE m^{-2} s^{-1} and 12 h photoperiod and then transferred to 2,000 µE m^{-2} s^{-1} for 24 hours, continuously. Reference plants were kept at 400 µE m^{-2} s^{-1}, continuously. The photosynthetic parameters were obtained from 3 independent measures on different leaves (n=3). Error bars represent standard error (S.E.). Different capital letters mean significant differences between light regimes and different lowercase letters represent significant differences between plant lines, according to Tukey's test, $p \leq 0.05$.

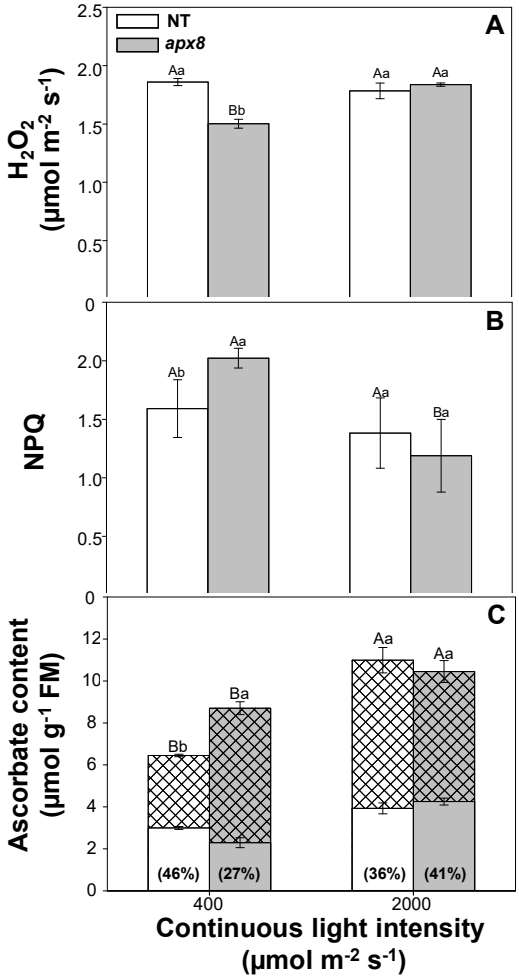

Figure 10. (A) Hydrogen peroxide content, (B) NPQ and (C) Ascorbate (ASC) and dehydroascorbate (DHA) content measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants grown at 400 µE m^{-2} s^{-1} and 12 h photoperiod and then transferred to 2,000 µE m^{-2} s^{-1} for 24 hours, continuously. Reference plants were kept at 400 µE m^{-2} s^{-1}, continuously. ASC and DHA values are expressed in µmol per gram of fresh matter (g^{-1} F.M.) and are average of 3 independent measures on extracts from different leaves (n=3). (H_2O_2) values are expressed in µmol per gram of fresh matter (g^{-1} F.M.) and are average of 3 independent measures on extracts from different leaves (n=3). The actinic light used in NPQ determination was 1000 µmol m^{-2} s^{-1}. Error bars represent standard error (S.E.; n=3). Different capital letters mean significant differences between light regimes and different lowercase letters represent significant differences between plant lines, according to Tukey's test, $p \leq 0.05$.

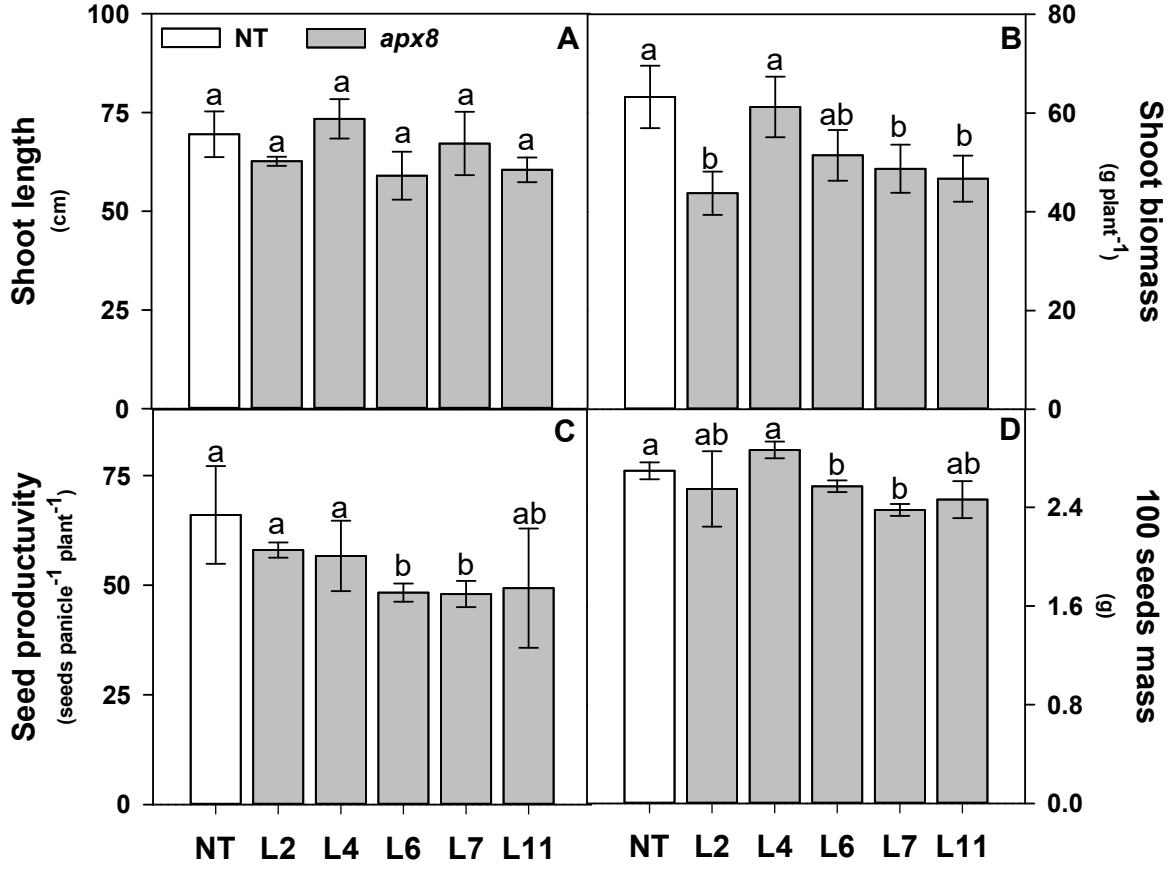

Figure S1. Phenotypic characterization of *apx8* silenced rice lines. Plants were cultivated under greenhouse conditions (30±5 °C, 50±20 % humidity, maximum PPFD average of 850 μmol^{-2} s^{-1} at noon and 12 hours of photoperiod) for 120 days. (A) Shoot length, measured from the colm basis to the top of highest leaf, (B) shoot biomass, (C) total seed productivity and (D) mass of 100 seeds. Columns represent average of 6 independent plants (n=6) and error bars means standard deviation. Different letters represent significant difference at 0.05 confidence level (Tukey's test) among rice lines.

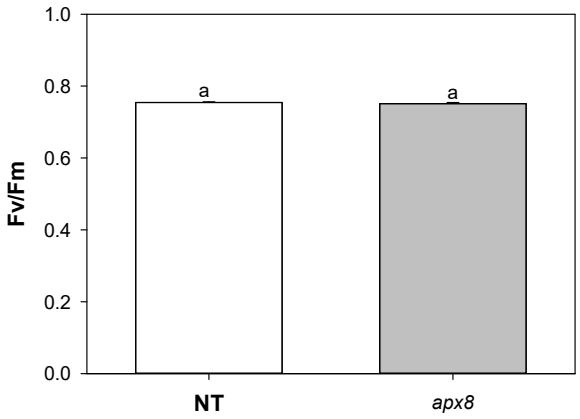

Figure S2. Maximum photosynthetic quantum efficiency (Fv/Fm) measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants were acclimated to 400 μE m^{-2} s^{-1} and 12 h photoperiod. Fv/Fm values are average of 24 independent measures on different leaves (n=24). Error bars represent standard error (S.E). Different letters represent significant difference between the two plant lines, analysed by Tukey's test, $p \leq 0.05$.

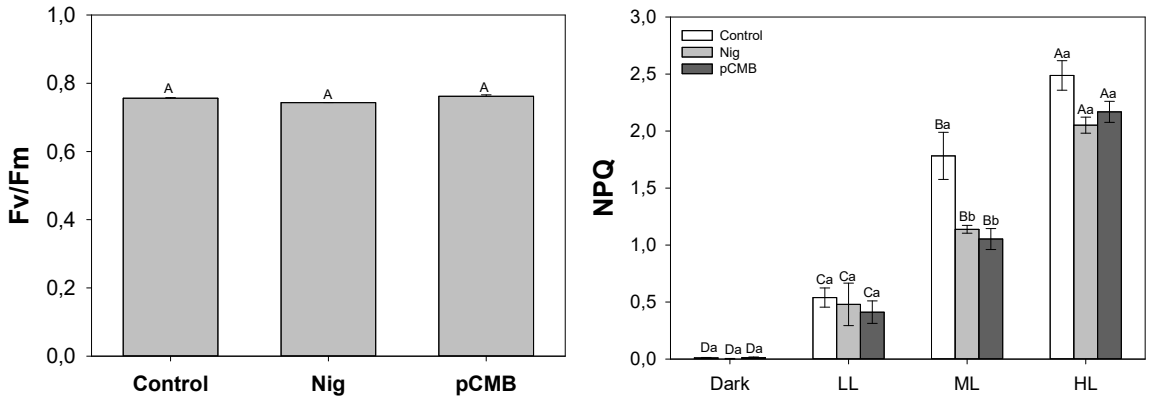

Figure S3. Maximum potential PSII quantum yield (Fv/Fm) and non-photochemical quenching (NPQ) measured in leaf segments from non-transformed (NT) rice plants. Plants grown at 400 µE m-2 s-1 and 12 h photoperiod. Leaf segments were infiltrated with 4 µM nigericin (NIG) or 30 µM pCMB (pCMB), inhibitors of ∆pH and APX activity, respectively. The values represent average of 3 independent assays on performed different leaves (n=3). Error bars represent standard error (S.E.). Different capital letters mean significant differences between light regimes and different lowercase letters represent significant differences between chemical treatments.

Table S1. State transition-related parameters determined on leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants grown at 400 μE m^{-2} s^{-1} and 12 h photoperiod. The values represent average of 8 independent determinations and ± values mean the standard error (n=8). The determinations were performed according to Ruban et al., (1999).

	qT	**qS**
NT	0.065±0.007	0.95±0.014
apx8	0.069±0.003	0.96±0.011

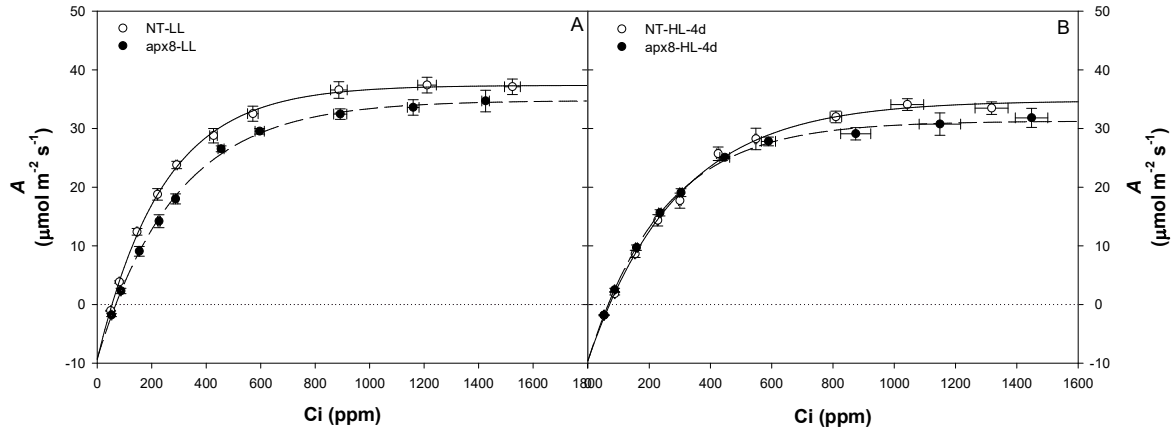

Figure S4. Photosynthesis-CO_2 fitted curves (A-Ci) measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants grown at 400 µE m^{-2} s^{-1} and 12 h photoperiod and then transferred to 1,800 µE m^{-2} s^{-1} for 4 days (HL-4d). Photosynthetic values are expressed in µmol CO_2 m^{-2} s^{-1} and are average of 3 independent measures from different leaves (n=3). Error bars represent standard error (S.E.).

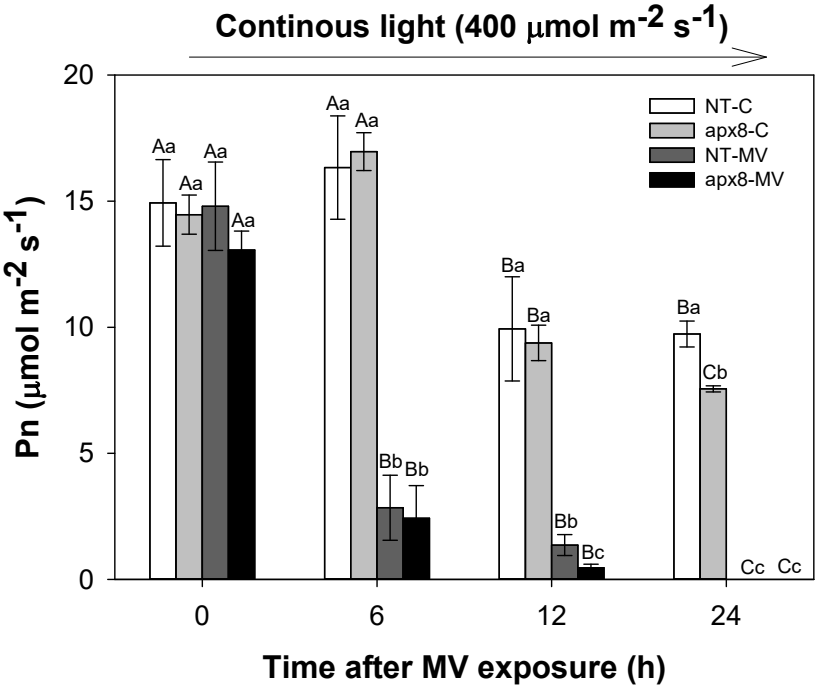

Figure S5. Photosynthetic CO_2 assimilation (Pn) measured in leaves from non-transformed (NT) and thylakoidal ascorbate peroxidase 8 silenced (*apx8*) rice plants. Plants grown at 400 μmol m^{-2} s^{-1} and 12 h photoperiod and then were sprayed with a solution containing 125 μM methyl-viologen (MV) and 0.01% Triton-X 100 (v/v). The Pn values were noted after 0, 6 12 and 24 hours of MV exposure and are average of 3 independent measures on different leaves (n=3). During MV treatment, the plants were exposed to continuous light (400 μE m^{-2} s^{-1}). Error bars represent standard error (S.E.). Different capital letters means significant differences between time of MV exposure and different lowercase letters represent significant differences between plant lines/MV concentrations.

CONCLUSION

CONCLUSION

The data reported in the current doctoral thesis allow reaching the following main conclusions:

1. Use of the new fluorimetric-based methodology described by Ruban and Murchie (2012), which is related to photochemical quenching quantification in the dark (qPd), is an effective methodology for photoinhibition monitoring.
2. New qPd methodology was successfully employed in different plant species (*A. thaliana* and *O. sativa*) and distinct physiological contexts, such as ontogenetic variations, different light regimes and increased chloroplastic H_2O_2 production (silenced *apx8* plants), corroborating its great potential.
3. Photoprotection and qPd stability is closely related to efficiency of pNPQ formation. These results were evidenced in crops for the first time.
4. Experimental data suggest a close relation between pNPQ efficiency and photosynthetic pigments content, especially chlorophyll and carotenoids. In addition, pNPQ parameter apparently is dependent on antioxidative metabolism.
5. Accordingly, rice plants silenced for thylakoidal APX acclimated to moderate light conditions exhibit higher ROS accumulation, reduced chlorophyll content and are more susceptible to photoinhibition than NT plants. These features might have been related to impaired growth and productivity.
6. Interestingly, *apx8* plants still capable of acclimation to high light conditions. This ability probably was related with ROS scavenging compensatory mechanisms triggering, displaying normal chlorophyll contents and NPQ formation.

APPENDICES

APPENDICES

The following articles were published (accepted) during the period of doctoral course (2013-2016) and are very relevant for scientific formation of the candidate:

1. **Carvalho, F.E.L.**, Ribeiro, C.W., Martins, M.O., Bonifacio, A., Staats, C.C., Andrade, C.M.B., Cerqueira, J.V., Margis-Pinheiro, M., Silveira, J.A.G., 2014. Cytosolic APX knockdown rice plants sustain photosynthesis by regulation of protein expression related to photochemistry, Calvin cycle and photorespiration. Physiol Plant 150, 632–645. doi:10.1111/ppl.12143
2. Caverzan, A., Bonifacio, A., **Carvalho, F.E.L.**, Andrade, C.M.B., Passaia, G., Schünemann, M., Maraschin, F. dos S., Martins, M.O., Teixeira, F.K., Rauber, R., Margis, R., Silveira, J.A.G., Margis-Pinheiro, M., 2014. The knockdown of chloroplastic ascorbate peroxidases reveals its regulatory role in the photosynthesis and protection under photo-oxidative stress in rice. Plant Sci 214, 74–87. doi:10.1016/j.plantsci.2013.10.001
3. Silveira, J.A.G., **Carvalho, F.E.L.**, 2016. Proteomics, photosynthesis and salt resistance in crops: An integrative view. J. Proteomics doi:10.1016/j.jprot.2016.03.013
4. Passaia, G., Caverzan, A., Fonini, L.S., **Carvalho, F.E.L.**, Silveira, J.A.G., Margis-Pinheiro, M., 2014. Chloroplastic and mitochondrial GPX genes play a critical role in rice development. Biol Plant 4–7. doi:10.1007/s10535-014-0394-9
5. Sousa, R.H. V., **Carvalho, F.E.L.**, Ribeiro, C.W., Passaia, G., Cunha, J.R., Lima-Melo, Y., Margis-Pinheiro, M., Silveira, J.A.G., 2015. Peroxisomal APX knockdown triggers antioxidant mechanisms favourable for coping with high photorespiratory H_2O_2 induced by CAT deficiency in rice. Plant Cell Environ 38, 499–513. doi:10.1111/pce.12409
6. Lima-Melo, Y., **Carvalho, F.E.L.**, Martins, M.O., Passaia, G., Sousa, R.H. V, Lima Neto, M.C., Margis-Pinheiro, M., Silveira, J.A.G., 2016. Mitochondrial GPX1 silencing triggers differential photosynthesis impairment in response to salinity in rice plants. J Integr Plant Biol n/a–n/a. doi:10.1111/jipb.12464
7. Ponte, L.F.A., Silva, A.L.C., **Carvalho, F.E.L.**, Maia, J.M., Voigt, E.L., Silveira, J.A.G., 2014. Salt-induced delay in cotyledonary globulin mobilization is abolished by induction of proteases and leaf growth sink strength at late seedling establishment in cashew. J Plant Physiol 171, 1362–1371. doi:10.1016/j.jplph.2014.06.001
8. Lima, C.S., Ferreira-Silva, S.L., Silva, E.N., Aragão, R.M., Lima Neto, M.C., **Carvalho, F.E.L.** and Silveira, J.A.G., 2016. Coordinated photosynthetic and antioxidant mechanisms confer photoprotection against extreme condition of drought combined with high light in cashew plants. Tree Physiol. Accepted manuscript (Major revision)
9. Bonifacio, A., **Carvalho, F.E.L.**, Martins, M.O., Lima Neto, M.C., Cunha, J.R., Ribeiro, C.W., Margis-Pinheiro, M., Silveira, J.A.G., 2016. Silenced rice in both cytosolic ascorbate peroxidases displays pre-acclimation to cope with oxidative stress induced by 3-aminotriazole inhibited catalase. J Plant Physiol Accepted manuscript (Minor revision)
10. Cunha, J.R., Lima Neto, M.C., **Carvalho, F.E.L.**, Martins, M.O., Jardim-Messeder, D., Margis-Pinheiro, M., Silveira, J.A.G., 2016. Salinity and osmotic stress trigger different antioxidant responses related to cytosolic ascorbate peroxidase knockdown in rice roots. Environ Exp Bot Accepted manuscript (Major revision)

Physiologia Plantarum
An International Journal for Plant Biology

Physiologia Plantarum 150: 632–645. 2014
 ISSN 0031-9317

Cytosolic APX knockdown rice plants sustain photosynthesis by regulation of protein expression related to photochemistry, Calvin cycle and photorespiration

Fabrício E. L. Carvalho[a], Carolina W. Ribeiro[b], Márcio O. Martins[a], Aurenivia Bonifacio[a], Charley C. Staats[c], Cláudia M. B. Andrade[c], João V. Cerqueira[a], Márcia Margis-Pinheiro[b] and Joaquim A. G. Silveira[a,*]

[a]Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza, CE, Brazil
[b]Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[c]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

Correspondence
*Corresponding author,
e-mail: silveira@ufc.br

Received 26 October 2013;
revised 27 November 2013

doi:10.1111/ppl.12143

The biochemical mechanisms underlying the involvement of cytosolic ascorbate peroxidases (cAPXs) in photosynthesis are still unknown. In this study, rice plants doubly silenced in these genes (APX1/2) were exposed to moderate light (ML) and high light (HL) to assess the role of cAPXs in photosynthetic efficiency. APX1/2 mutants that were exposed to ML overexpressed seven and five proteins involved in photochemical activity and photorespiration, respectively. These plants also increased the pheophytin and chlorophyll levels, but the amount of five proteins that are important for Calvin cycle did not change. These responses in mutants were associated with Rubisco carboxylation rate, photosystem II (PSII) activity and potential photosynthesis, which were similar to non-transformed plants. The upregulation of photochemical proteins may be part of a compensatory mechanism for APX1/2 deficiency but apparently the finer-control for photosynthesis efficiency is dependent on Calvin cycle proteins. Conversely, under HL the mutants employed a different strategy, triggering downregulation of proteins related to photochemical activity, Calvin cycle and decreasing the levels of photosynthetic pigments. These changes were associated to strong impairment in PSII activity and Rubisco carboxylation. The upregulation of some photorespiratory proteins was maintained under that stressful condition and this response may have contributed to photoprotection in rice plants deficient in cAPXs. The data reveal that the two cAPXs are not essential for photosynthesis in rice or, alternatively, the deficient plants are able to trigger compensatory mechanisms to photosynthetic acclimation under ML and HL conditions. These mechanisms involve differential regulation in protein expression related to photochemistry, Calvin cycle and photorespiration.

Abbreviations – APX1/2 , cytosolic APX double silenced rice plants; cAPX, cytosolic ascorbate peroxidases; CEF, cyclic electron flux; CSD2, Cu/Zn-SOD; ETR, actual flux of electrons from the PSII; FNR, ferredoxin NADP(H) oxireductase; G3PD, glyceraldehyde-3-phosphate dehydrogenase; GLDC, glycine decarboxylase; GO, glycolate oxidase; GS, glutamine synthetase; HL, high light; Lhcb1, chlorophyll *a*/*b* binding protein type 1; ML, moderate light; NPQ, non-photochemical quenching; NT, non-transformed rice plants; PGK, phosphoglycerate kinase; PSII, photosystem II; qP, photochemical quenching; qRT-PCR, quantitative real-time polymerase chain reaction; RLS, Rubisco larger subunit; RuBP, ribulose-1,5-biphosphate.

Plant Science 214 (2014) 74–87

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

The knockdown of chloroplastic ascorbate peroxidases reveals its regulatory role in the photosynthesis and protection under photo-oxidative stress in rice

Andréia Caverzan[a,b], Aurenivia Bonifacio[d], Fabricio E.L. Carvalho[d], Claudia M.B. Andrade[b,1], Gisele Passaia[a], Mariana Schünemann[a], Felipe dos Santos Maraschin[c], Marcio O. Martins[d], Felipe K. Teixeira[a], Rafael Rauber[b], Rogério Margis[b,e], Joaquim Albenisio Gomes Silveira[d], Márcia Margis-Pinheiro[a,*]

[a] Department of Genetics, Federal University of Rio Grande do Sul, Brazil
[b] Biotechnology Center, Federal University of Rio Grande do Sul, Brazil
[c] Department of Botany, Federal University of Rio Grande do Sul, Brazil
[d] Department of Biochemistry and Molecular Biology, Federal University of Ceará, Brazil
[e] Department of Biophysics, Federal University of Rio Grande do Sul, Brazil

ARTICLE INFO

Article history:
Received 19 July 2013
Received in revised form
12 September 2013
Accepted 1 October 2013
Available online 8 October 2013

Keywords:
Oryza sativa
Rice
APX
Ascorbate peroxidase
Photosynthesis
Oxidative stress

ABSTRACT

The inactivation of the chloroplast ascorbate peroxidases (chlAPXs) has been thought to limit the efficiency of the water–water cycle and photo-oxidative protection under stress conditions. In this study, we have generated double knockdown rice (*Oryza sativa* L.) plants in both *OsAPX7* (sAPX) and *OsAPX8* (tAPX) genes, which encode chloroplastic APXs (chlAPXs). By employing an integrated approach involving gene expression, proteomics, biochemical and physiological analyses of photosynthesis, we have assessed the role of chlAPXs in the regulation of the protection of the photosystem II (PSII) activity and CO_2 assimilation in rice plants exposed to high light (HL) and methyl viologen (MV). The chlAPX knockdown plants were affected more severely than the non-transformed (NT) plants in the activity and structure of PSII and CO_2 assimilation in the presence of MV. Although MV induced significant increases in pigment content in the knockdown plants, the increases were apparently not sufficient for protection. Treatment with HL also caused generalized damage in PSII in both types of plants. The knockdown and NT plants exhibited differences in photosynthetic parameters related to efficiency of utilization of light and CO_2. The knockdown plants overexpressed other antioxidant enzymes in response to the stresses and increased the GPX activity in the chloroplast-enriched fraction. Our data suggest that a partial deficiency of chlAPX expression modulate the PSII activity and integrity, reflecting the overall photosynthesis when rice plants are subjected to acute oxidative stress. However, under normal growth conditions, the knockdown plants exhibit normal phenotype, biochemical and physiological performance.

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; cAPX, cytosol APX; CAT, catalase; chlAPX, chloroplastic APX; Ci, intercellular concentration of CO_2; E, transpiration; ETR, apparent electron transport rate; EXC, energy excess; F_v/F_m, potential quantum yield of photosystem II; GPX, glutathione peroxidase; g_s, stomatal conductance; H_2O_2, hydrogen peroxide; HL, high light; J_{max}, maximum photosynthetic electron transport; mAPX, peroxisome/glyoxysomes APX; miAPX, mitochondria APX; MV, methyl viologen; NPQ, non-photochemical quenching; NT, non-transformed; PET, photosynthetic electron transport; P_N–Ci, photosynthesis depending on the intercellular concentration of CO_2; P_N–PPFD, photosynthesis depending on light intensity; P_N, net CO_2 assimilation; PPFD, photosynthetic photon flux density; PQ, plastoquinone pool; P_r, photorespiration; PSI, photosystem I; PSII, photosystem II; qP, photochemical quenching; R_d, light respiration; R_n, dark respiration; ROS, reactive oxygen species; RT-qPCR, quantitative real-time PCR; sAPX, stroma APX; SOD, superoxide dismutase; tAPX, thylakoid APX; TBARS, thiobarbituric acid-reactive substances; V_{cmax}, maximum Rubisco carboxylation rate; $\Delta F_v/F'_m$, actual quantum yield of photosystem II.

* Corresponding author at: Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, CEP: 915910-970, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 9814.
E-mail addresses: marcia.margis@ufrgs.br, marcia.p.margis@gmail.com (M. Margis-Pinheiro).
[1] Present address: Department of Chemistry, Federal University of Mato Grosso, Brazil.

0168-9452/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.plantsci.2013.10.001

ARTICLE IN PRESS

JPROT-02452; No of Pages 12

Journal of Proteomics xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Review

Proteomics, photosynthesis and salt resistance in crops: An integrative view

Joaquim A.G. Silveira *, Fabricio E.L. Carvalho

Department of Biochemistry and Molecular Biology, Laboratory of Plant Metabolism, Federal University of Ceara, Fortaleza CEP 60451-970, Brazil

ARTICLE INFO

Article history:
Received 29 December 2015
Received in revised form 12 February 2016
Accepted 5 March 2016
Available online xxxx

Keywords:
Phosphoproteomics
Photosynthesis proteins
Redox proteomics
Thiol proteomics
Salinity

ABSTRACT

Salinity is a stressful condition that causes a significant decrease in crop production worldwide. Salt stress affects several photosynthetic reactions, including the modulation of several important proteins. Despite these effects, few molecular-biochemical markers have been identified and evaluated for their importance in improving plant salt resistance. Proteomics is a powerful tool that allows the analysis of multigenic events at the post-translational level that has been widely used to evaluate protein modulation changes in plants exposed to salt stress. However, these studies are frequently fragmented and the results regarding photosynthesis proteins in response to salinity are limited. These constraints could be related to the low number of important photosynthetic proteins differently modulated in response to salinity, as has been commonly revealed by conventional proteomics. In this review, we present an evaluation and perspective on the integrated application of proteomics for the identification of photosynthesis proteins to improve salt resistance. We propose the use of phospho-, thiol- and redox-proteomics, associated with the utilization of isolated chloroplasts or photosynthetic sub-organellar components. This strategy may allow the characterization of essential proteins, providing a better understanding of photosynthesis regulation. Furthermore, this may contribute to the selection of molecular markers to improve salt resistance in crops.

Contents

1. Introduction

During the last few years, numerous studies have been published involving the utilization of proteomics techniques to evaluate the modulation of several proteins in response to salt stress in a diverse variety of crops [1]. Studies in intact leaves have involved proteins related to various important physiological processes, such as photosynthesis, energetic, primary metabolism, antioxidant proteins and others [2]. Photosynthesis is one of the most important processes related to crop productivity and is extremely sensitive to salt stress. Paradoxically, few studies have been published involving photosynthetic protein modulation to improve salt resistance. Unexpectedly, although proteomics is

* Corresponding author.
E-mail addresses: silveira@ufc.br (J.A.G. Silveira), fabricioeulalio@hotmail.com (F.E.L. Carvalho).

http://dx.doi.org/10.1016/j.jprot.2016.03.013
1874-3919/

Please cite this article as: J.A.G. Silveira, F.E.L. Carvalho, Proteomics, photosynthesis and salt resistance in crops: An integrative view, J Prot (2016), http://dx.doi.org/10.1016/j.jprot.2016.03.013

doi: 10.1007/s10535-014-0394-9 BIOLOGIA PLANTARUM XX (X): XXX-XXX, 20XX

BRIEF COMMUNICATION

Chloroplastic and mitochondrial *GPX* genes play a critical role in rice development

G. PASSAIA[1], A. CAVERZAN[1,2], L.S. FONINI[1,2], F.E.L. CARVALHO[3], J.A.G. SILVEIRA[3], and M. MARGIS-PINHEIRO[1]*

Department of Genetics, Federal University of Rio Grande do Sul, Rio Grande do Sul, 15053 RS, Brazil[1]
Biotechnology Center, Federal University of Rio Grande do Sul, Rio Grande do Sul, 15053 RS, Brazil[2]
Department of Biochemistry and Molecular Biology, Federal University of Ceará, Ceará, 60020 CE, Brazil[3]

Abstract

Plant glutathione peroxidases (GPX) catalyze the reduction of H_2O_2 or organic hydroperoxides to water, mitigating the toxicity of these compounds to cells. In rice plants, the *GPX* gene family is composed of five members that are distributed in a range of sub-cellular compartments, including cytosol, mitochondria, chloroplasts, or endoplasmic reticulum. Of these, *OsGPX1* and *OsGPX4* are located in mitochondria and chloroplasts, respectively. To understand the role of these *GPX* in rice, the effect of knockdown of *OsGPX1* and *OsGPX4* in rice plants was evaluated. Our data show that *OsGPX4* was essential for *in vitro* rice regeneration because no plants were obtained from calli carrying a hairpin construct against *OsGPX4*. Although the knockdown of *OsGPX1* did not impair plant regeneration, the plants with silenced *OsGPX1* (*GPX1s* plants) showed reduced shoot length and a reduced number of seeds compared to the non-transformed rice plants. These results indicate that OsGPX1 and OsGPX4 are essential for redox homeostasis which leads to normal growth and development in rice.

Additional key words: glutathione peroxidase, hairpin construct, *in vitro* regeneration, *Oryza sativa*, oxidative stress, seed production, transgenic plants.

Reactive oxygen species (ROS), which are generated during normal plant development, act as signaling molecules and regulate essential processes. They are generated in toxic concentrations during biotic and abiotic stresses (Halliwell *et al.* 2006, Faltin *et al.* 2010). To maintain redox homeostasis, ROS-scavenging systems use thiol-containing proteins as redox transducers (Foyer and Noctor 2005) as well as ascobate peroxidase (APX), catalase (CAT), superoxide dimustase (SOD), and non-enzymatic compounds ascorbate (Asc), glutathione (GSH), tocopherols, and carotenoids (Koh *et al.* 2007). Plant glutathione peroxidases (GPX) preferentially use thioredoxin as electron donor to catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols (Herbette *et al.* 2007). Contrary to most plant GPX, mammalian isoforms carry seleno-cysteine in their active site, allowing them to preferentially use glutathione as electron donor (Herbette *et al.* 2007). In plants, GPXs are involved in biotic and abiotic stress responses (Milla *et al.* 2003, Navrot *et al.* 2006, Xu *et al.* 2012, Zhang *et al.* 2012). For example, in *Arabidopsis thaliana*, a GPX loss or gain of function mutants demonstrated the role of these enzymes in H_2O_2 scavenging, signal transduction, photo-oxidative stress tolerance, and nuclear DNA damage protection (Miao *et al.* 2006, Chang *et al.* 2009, Gaber *et al.* 2012). To understand the role of GPX in rice, we tried to obtain transgenic rice plants that were silenced for the whole *GPX* family. In the current study, we present the characteristics of *OsGPX1* or *OsGPX4* rice knock-down transgenic plants. The transformation of rice calli with a hairpin construct targeted to chloroplastic *OsGPX4*

Received 4 February 2013, *accepted* 18 October 2013.
Abbreviations: GPX - glutathione peroxidase; qPCR - quantitative polymerase chain reaction.
Acknowledgments: This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and the International Centre for Genetic Engineering and Biotechnology (ICGEB).
* Corresponding author: fax: (+55) 51 3308-9814, e-mail: marcia.margis@ufrgs.br

Plant, Cell & Environment

Plant, Cell and Environment (2014) doi: 10.1111/pce.12409

Original article

Peroxisomal APX knockdown triggers antioxidant mechanisms favourable for coping with high photorespiratory H_2O_2 induced by CAT deficiency in rice

Rachel H. V. Sousa[1], Fabricio E. L. Carvalho[1], Carol W. Ribeiro[2], Gisele Passaia[2], Juliana R. Cunha[1], Yugo Lima-Melo[1], Márcia Margis-Pinheiro[2] & Joaquim A. G. Silveira[1]

[1]*Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza, CE 60440-900, Brazil and*
[2]*Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 91501970, Brazil*

ABSTRACT

The physiological role of peroxisomal ascorbate peroxidases (pAPX) is unknown; therefore, we utilized pAPX4 knockdown rice and catalase (CAT) inhibition to assess its role in CAT compensation under high photorespiration. pAPX4 knockdown induced co-suppression in the expression of pAPX3. The rice mutants exhibited metabolic changes such as lower CAT and glycolate oxidase (GO) activities and reduced glyoxylate content; however, APX activity was not altered. CAT inhibition triggered different changes in the expression of CAT, APX and glutathione peroxidase (GPX) isoforms between non-transformed (NT) and silenced plants. These responses were associated with alterations in APX, GPX and GO activities, suggesting redox homeostasis differences. The glutathione oxidation-reduction states were modulated differently in mutants, and the ascorbate redox state was greatly affected in both genotypes. The pAPX suffered less oxidative stress and photosystem II (PSII) damage and displayed higher photosynthesis than the NT plants. The improved acclimation exhibited by the pAPX plants was indicated by lower H_2O_2 accumulation, which was associated with lower GO activity and glyoxylate content. The suppression of both pAPXs and/or its downstream metabolic and molecular effects may trigger favourable antioxidant and compensatory mechanisms to cope with CAT deficiency. This physiological acclimation may involve signalling by peroxisomal H_2O_2, which minimized the photorespiration.

Key-words: *Oryza sativa*; ascorbate peroxidase; H_2O_2 homeostasis; oxidative stress; photorespiration; signalling.

INTRODUCTION

Despite the great advances in the understanding of oxidative metabolism in plants in the last decades (to review see Foyer & Noctor 2011), several contradictory data involving the classical antioxidant pathways have frequently been reported (Rizhsky *et al.* 2002; Miller *et al.* 2007; Koussevitzky *et al.* 2008; Carvalho *et al.* 2014). In addition, the physiological role displayed by some ascorbate peroxidase (APX) isoforms, especially peroxisomal isoforms, is poorly known. APX is coded by a gene family that is widely represented in plants, and the existence of two peroxisomal APX (pAPX) isozymes, which are targeted for bind to the external surface of peroxisomal membranes, has been confirmed in *Arabidopsis* (Panchuk *et al.* 2002; Narendra *et al.* 2006). A third isoform possibly targeted to the peroxisomal matrix has also been reported (Panchuk *et al.* 2002). These isoforms were initially characterized many years ago (Yamaguchi *et al.* 1995; Bunkelmann & Trelease 1996; Mullen *et al.* 1999). In rice, two peroxisomal APX isoforms, *OsAPX3* and *OsAPX4*, have been characterized, and both enzymes are also targeted for membranes (Teixeira *et al.* 2004, 2006).

The most important remaining questions concern the importance of pAPX in H_2O_2 scavenging during photorespiration and its possible role in signalling and peroxisome-cytosol cross-talking. The results reported to date remain controversial, but suggest that pAPX is important in the absence or in complement to catalase (CAT) (Wang *et al.* 1999). Works in which pAPX was overexpressed in *Arabidopsis* have suggested that transgenic plants are more resistant to certain abiotic stresses (Kavitha *et al.* 2008), oxidative stress generated by CAT inhibition (Wang *et al.* 1999) and methyl viologen (Kavitha *et al.* 2008). Tobacco transgenic plants that overexpress pAPX from *Salicornia brachiata* have displayed higher seed germination under osmotic and saline conditions (Singh *et al.* 2014). Nevertheless, a study conducted with pAPX3 knockout (KO) *Arabidopsis* grown under normal growth conditions and exposed to salt, chilling and heat stresses clearly showed that this gene or the encoded protein is dispensable for plant growth and development (Narendra *et al.* 2006).

Peroxisomal APX isoenzymes may act in concert with other peroxidases in peroxisomes, especially CAT. High photorespiratory H_2O_2 production in C3 plants subjected to abiotic stress is common, and under extreme situations, the photorespiration might represent up to 50% of Rubisco activity (Peterhansel & Maurino 2011). CAT has a high K_M for H_2O_2, whereas pAPX has high affinity for this substrate (Yamaguchi *et al.* 1995). The simultaneous activities of both enzymes are important because they allow the elimination of

Correspondence: J. A. G. Silveira. Fax: 55 085-33669789; e-mail: silveira@ufc.br

JIPB Journal of Integrative Plant Biology

Mitochondrial GPX1 silencing triggers differential photosynthesis impairment in response to salinity in rice plants

Yugo Lima-Melo[1], Fabricio E. L. Carvalho[1], Márcio O. Martins[1], Gisele Passaia[2], Rachel H. V. Sousa[1], Milton C. Lima Neto[1], Márcia Margis-Pinheiro[2] and Joaquim A. G. Silveira[1]*

[1]Department of Biochemistry and Molecular Biology, Federal University of Ceará, CEP 60451-970, Fortaleza, Ceará, Brazil, [2]Department of Genetics, Federal University of Rio Grande do Sul, CEP 91501-970, Porto Alegre, Rio Grande do Sul, Brazil. *Correspondence: silveira@ufc.br

Research Article

Abstract The physiological role of plant mitochondrial glutathione peroxidases is scarcely known. This study attempted to elucidate the role of a rice mitochondrial isoform (GPX1) in photosynthesis under normal growth and salinity conditions. GPX1 knockdown rice lines (GPX1s) were tested in absence and presence of 100 mM NaCl for 6 d. Growth reduction of GPX1s line under non-stressful conditions, compared with non-transformed (NT) plants occurred in parallel to increased H_2O_2 and decreased GSH contents. These changes occurred concurrently with photosynthesis impairment, particularly in Calvin cycle's reactions, since photochemical efficiency did not change. Thus, *GPX1* silencing and downstream molecular/metabolic changes modulated photosynthesis differentially. In contrast, salinity induced reduction in both phases of photosynthesis, which were more impaired in silenced plants. These changes were associated with root morphology alterations but not shoot growth. Both studied lines displayed increased GPX activity but H_2O_2 content did not change in response to salinity. Transformed plants exhibited lower photorespiration, water use efficiency and root growth, indicating that GPX1 could be important to salt tolerance. Growth reduction of GPX1s line might be related to photosynthesis impairment, which in turn could have involved a cross talk mechanism between mitochondria and chloroplast originated from redox changes due to GPX1 deficiency.

Keywords: CO_2 assimilation; glutathione peroxidase; *Oryza sativa*; photochemistry; redox homeostasis

Citation: Lima-Melo Y, Carvalho FEL, Martins MO, Passaia G, Sousa RHV, Neto MCL, Margis-Pinheiro M, Silveira JAG (2016) Mitochondrial GPX1 silencing triggers differential photosynthesis impairment in response to salinity in rice plants. **J Integr Plant Biol** XX:XX–XX doi: 10.1111/jipb.12464

Edited by: Miguel A. Piñeros, United States Department of Agriculture-Agricultural Research Service, Cornell University, USA

Received Dec. 22, 2015; **Accepted** Jan. 19, 2016

Available online on Jan. 22, 2016 at www.wileyonlinelibrary.com/journal/jipb

INTRODUCTION

Glutathione peroxidases (GPX, EC 1.11.1.9) are largely distributed in animal cells, where they play several important physiological roles such as H_2O_2 scavenging, maintaining of cellular redox homeostasis and membrane protection against peroxidation (Herbette et al. 2007). Plants also have a large family of GPX isoforms distributed in virtually all cellular compartments (Margis et al. 2008; Passaia and Margis-Pinheiro 2015). Differently from animal GPX, the physiological role of these peroxidases in plants is less known (Faltin et al. 2010; Gaber et al. 2012; Zhai et al. 2013). Despite the several experimental evidences that have suggested that plant GPX family displays some similar roles of those played in animals, the physiological functions of specific isoforms are still unknown (Passaia et al. 2014a; Passaia and Margis-Pinheiro 2015). These enzymes might act as antioxidants in the elimination of H_2O_2, organic hydroperoxides and/or lipid peroxides (Faltin et al.

Abbreviations

A	CO_2 assimilation rate
A_{max}	maximum photosynthetic rate
C_i	intercellular CO_2 partial pressure
E	leaf transpiration
ETR	actual electron transport rate from PSII
F_v/F_m	maximum quantum yield of PSII
GPX	glutathione peroxidase
g_s	stomatal conductance
GSH	reduced glutathione
J_{max}	maximum electron transport rate
L_s	stomatal limitation
L_m	metabolic limitation
NPQ	non-photochemical quenching
PPFD	photosynthetic photon flux density
P_r	photorespiration
PSII	photosystem II
qP	photochemical quenching
R_d	dark respiration
ROS	reactive oxygen species
TBARS	thiobarbituric acid reactive substances
Vc_{max}	maximum carboxylation rate of Rubisco
WUE	water use efficiency
α	quantum efficiency
$\Delta F/F_m'$	effective quantum yield of PSII

Journal of Plant Physiology 171 (2014) 1362–1371

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

Salt-induced delay in cotyledonary globulin mobilization is abolished by induction of proteases and leaf growth sink strength at late seedling establishment in cashew

Luiz Ferreira Aguiar Ponte[a], André Luis Coelho da Silva[b], Fabrício Eulálio Leite Carvalho[b], Josemir Moura Maia[c], Eduardo Luiz Voigt[d], Joaquim Albenisio Gomes Silveira[b,*]

[a] Centro de Ciências Agrárias e Biológicas, Universidade Estadual Vale do Acaraú, CEP 62040-370, Sobral, CE, Brazil
[b] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, CEP 60451-970, Fortaleza, Ceará, Brazil
[c] Campos IV – Catolé do Rocha, Centro de Ciências Humanas e Agrárias, CCHA, Universidade Estadual da Paraiba, Paraiba, Brazil
[d] Laboratório de Estudos em Biotecnologia Vegetal, Departamento de Biologia Celular e Genética, Universidade Federal do Rio Grande do Norte, RN, Brazil

ARTICLE INFO

Article history:
Received 15 February 2014
Received in revised form 3 June 2014
Accepted 3 June 2014
Available online 24 June 2014

Keywords:
Anacardium occidentale
Plant establishment
Protein metabolism
Proteases
Salinity

ABSTRACT

Seedling establishment in saline conditions is crucial for plant survival and productivity. This study was performed to elucidate the biochemical and physiological mechanisms involved with the recovery and establishment of cashew seedlings subjected to salinity. The changes in the Na+ levels and K/Na ratios, associated with relative water content, indicated that osmotic effects were more important than salt toxicity in the inhibition of seedling growth and cotyledonary protein mobilization. Salinity (50 mM NaCl) induced a strong delay in protein breakdown and amino acid accumulation in cotyledons, and this effect was closely related to azocaseinolytic and protease activities. In parallel, proline and free amino acids accumulated in the leaves whereas the protein content decreased. Assays with specific inhibitors indicated that the most important proteases in cotyledons were of serine, cysteine and aspartic types. Proteomic analysis revealed that most of the cashew reserve proteins are 11S globulin-type and that these proteins were similarly degraded under salinity. In the late establishment phase, the salt-treated seedlings displayed an unexpected recovery in terms of leaf growth and N mobilization from cotyledon to leaves. This recovery coordinately involved a great leaf expansion, decreased amino acid content and increased protein synthesis in leaves. This response occurred in parallel with a prominent induction in the cotyledon proteolytic activity. Altogether, these data suggest that a source–sink mechanism involving leaf growth and protein synthesis may have acted as an important sink for reserve mobilization contributing to the seedling establishment under salinity. The amino acids that accumulated in the leaves may have exerted negative feedback to act as a signal for the induction of protease activity in the cotyledon. Overall, these mechanisms employed by cashew seedlings may be part of an adaptive process for the efficient rescue of cotyledonary proteins, as the cashew species originates from an environment with N-poor soil and high salinity.

Introduction

Seedling establishment is a physiological process that is essential to plant survival, especially under adverse conditions such as salinity and low availability of mineral nutrients in soils. Under these conditions, germination and cotyledonary reserve mobilization are delayed, and seedling growth is strongly diminished (Voigt et al., 2009). Salinity may disturb these processes by osmotic and ionic effects, but the seed water absorption affected by these processes is crucial for germination (Wierzbicka and Obidzińska, 1998; Thomas et al., 2010; Marques et al., 2013). The ionic or osmotic effects of salinity on germination and reserve mobilization are

Abbreviations: 2-DE, two dimensional electrophoresis; DAS, days after sowing; E-64, 10 μM trans-epoxysuccinyl-L-leucylamide (4-guanidine) butane; ESI, electrospray ionization; PEPS, pepstatin; PMSF, phenylmethylsulfonyl fluoride; Q-TOF, quadrupole time-of-flight; RWC, relative water content; SSP, seed storage protein; UA, unity of activity; Ψs, osmotic potential.

* Corresponding author at: Avenida Humberto Monte SN, Universidade Federal do Ceará – Campus do Pici, Departamento de Bioquímica e Biologia Molecular, Bl. 907, sala 1080, CEP 60451-970, Fortaleza, Ceará, Brazil. Tel.: +55 85 33669821; fax: +55 85 33669789.
E-mail address: silveira@ufc.br (J.A.G. Silveira).

http://dx.doi.org/10.1016/j.jplph.2014.06.001
0176-1617/

Accepted manuscript in Tree Physiology (Major revision)

OXFORD UNIVERSITY PRESS | Tree Physiology

Coordinated photosynthetic and antioxidant mechanisms confer photoprotection against extreme condition of drought combined with high light in cashew plants

Journal:	*Tree Physiology*
Manuscript ID	TP-2016-120
Manuscript Type:	Research Paper
Date Submitted by the Author:	06-Apr-2016
Complete List of Authors:	Lima, Cristina; Universidade Federal do Ceara, Departamento de Bioquímica e Biologia Molecular Ferreira-Silva, Sergio; Universidade Federal Rural de Pernambuco, Unidade Acadêmica de Serra Talhada, Programa de Pós Graduação em Produção Vegetal Silva, Evandro; Universidade Estadual do Ceara, Faculdade de Educação, Ciências e Letras do Sertão Central Aragão, Rafael; Universidade Federal do Ceara, Departamento de Bioquímica e Biologia Molecular Lima Neto, Milton; Universidade Estadual Paulista Julio de Mesquita Filho - Campus Experimental do Litoral Paulista Carvalho, Fabricio; Universidade Federal do Ceara, Departamento de Bioquímica e Biologia Molecular Silveira, Joaquim; Universidade Federal do Ceará, Departamento de Bioquímica e Biologia Molecular
Keywords:	Anacardium occidentale, photochemistry, Photo-oxidative stress, Photosynthesis, Redox metabolism, Water Deficit

http://mc.manuscriptcentral.com/tp

Accepted manuscript in Journal of Plant Physiology (Minor revision)

Elsevier Editorial System(tm) for Journal of Plant Physiology

Manuscript Draft

Manuscript Number: JPLPH-D-16-00027R1

Title: Silenced rice in both cytosolic ascorbate peroxidases displays pre-acclimation to cope with oxidative stress induced by 3-aminotriazole-inhibited catalase

Article Type: Research Paper

Section/Category: Physiology

Keywords: ascorbate peroxidase; hydrogen peroxide; Oryza sativa; photorespiration; photosynthesis; redox metabolism.

Corresponding Author: Prof. Joaquim A G Silveira, PhD

Corresponding Author's Institution: Universidade Federal do Ceará

First Author: Aurenivia Bonifacio, Ph.D.

Order of Authors: Aurenivia Bonifacio, Ph.D.; Fabricio Eulalio L Carvalho, M.Sc.; Marcio O Martins, Ph.D.; Milton C Lima Neto, Ph.D.; Juliana R Cunha, M.Sc.; Carolina W Ribeiro, Ph.D.; Marcia Margis-Pinheiro, Ph.D.; Joaquim A G Silveira, PhD

Abstract: The maintaining of H2O2 homeostasis and signaling mechanisms in plant subcellular compartments are greatly dependent on cytosolic ascorbate peroxidases (APX1 and APX2) and peroxisomal catalase (CAT) activities. APX1/2 knockdown plants were utilized in this study in order to clarify the role of increased cytosolic H2O2 levels as a signaling for trigger antioxidant defense against oxidative stress generated in peroxisomes after 3-aminotriazole-inhibited catalase (CAT). Before 3-AT supplying, silenced APX1/2 plants showed deep changes in oxidative and antioxidant profile in comparison to NT plants. After 3-AT supplying, APX1/2 plants triggered up-expression of genes belonging to APX (OsAPX7 and OsAPX8) and GPX families (OsGPX1, OsGPX2, OsGPX3 and OsGPX5), but in lower extent compared to NT plants. In addition, APX1/2 exhibited lower glycolate oxidase (GO) activity, higher CO2 assimilation, higher cellular integrity and higher oxidation of GSH, whereas the H2O2 and lipid peroxidation levels were maintained unchanged. These evidences indicate that redox pre-acclimation displayed by silenced rice contributed to cope with oxidative stress generate by 3-AT. We suggest that APX1/2 plants were able to trigger alternative oxidative and antioxidant mechanisms involving signaling by H2O2, allowing these plants to display effective physiological responses for protection against oxidative damage generated by 3-AT, compared to non-transformed plants.

Accepted manuscript in Environmental and Experimental Botany (Major revision)

Elsevier Editorial System(tm) for
Environmental and Experimental Botany
Manuscript Draft

Manuscript Number: EEB-D-16-00165R1

Title: Salinity and osmotic stress trigger different antioxidant responses related to cytosolic ascorbate peroxidase knockdown in rice roots

Article Type: Research Paper

Keywords: Ascorbate peroxidase; Ionic stress; Oryza sativa; Redox metabolism; Salt stress.

Corresponding Author: Prof. Joaquim A G Silveira, PhD

Corresponding Author's Institution: Universidade Federal do Ceará

First Author: Juliana R Cunha, M.Sc.

Order of Authors: Juliana R Cunha, M.Sc.; Milton C Lima Neto, Ph.D.; Fabricio Eulálio L Carvalho, M.Sc.; Marcio O Martins, Ph.D.; Douglas Jardim-Messeder, M.Sc.; Marcia Margis-Pinheiro, Ph.D.; Joaquim A G Silveira, PhD

Abstract: Salinity and osmotic stress trigger distinct signals in roots, which might induce differences in antioxidant responses. To clarify these relationships, transgenic rice plants silenced in both cytosolic ascorbate peroxidases (apx1/2) and non-transformed (NT) were exposed to iso-osmotic concentrations of NaCl and mannitol. Under both stress conditions, apx1/2 roots did not suffer oxidative stress, revealing that cytosolic APXs were not crucial to oxidative protection. Silenced and non-transformed roots triggered different responses to high salinity and osmotic stress and these stressful factors induced also distinct antioxidant changes. High salinity up-regulated expression of important OsAPX isoforms and these changes were related to increased APX activity, especially in NT roots. Intriguingly, salt stress triggered up-regulation of OsCAT isoforms but CAT activity did not change in both genotypes. In contrast, mannitol trigged very low increment in expression of OsAPX isoforms but induced substantial up-regulation in APX activity in NT roots. Mannitol also remarkably up-regulated OsCATB expression in parallel to CAT activity, in both apx1/2 and NT roots. POD and GPX (glutathione peroxidases) activities were strongly increased by high salinity but did not change in response to mannitol, in both genotypes. The two stress types as well as apx1/2 and NT roots displayed different response in terms of modulation in the H2O2 levels but lipid peroxidation did not change. Membrane integrity was drastically affected by both stressful factors and similarly in both genotypes, whereas root fresh matter was affected only by salt stress. Altogether, the obtained data reveal that high salinity and osmotic stress trigger different antioxidant responses and these strategies were genotype-dependent. The different antioxidant molecular-biochemical mechanisms employed by cytosolic APX knockdown and non-transformed roots allowed reaching similar physiological performance.